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Characterisation of *var* genes and of the PfEMP-1 binding capacity to ICAM-1 of *Plasmodium falciparum* isolates from Indonesia

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapy
ATS	Acidic Terminal Sequence
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CIDR	Cysteine-rich Interdomain Regions
CR1	Complement Receptor 1
CSA	Chondroitin sulphate A
DBL	Duffy Binding-like
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
EBA	Erythrocytes Binding Antigen
EDTA	Ethylenediaminetetraacetic Acid
ELAM	Endothelial Leukocyte Adhesion Molecule
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GLRUP	Glutamate-Rich Protein
HA	Hyaluronic Acid
HB	Homology Block
HS	Heparan Sulphate
ICAM-1	Intercellular Adhesion Molecule-1
IE	Infected Erythrocyte
IFNγ	Interferon γ
IPTG	Isopropylthio-β-galactoside
KAHRP	Knob-associated Histidine-rich Protein
MOI	Multiplicity of Infection
MSP	Merozoite Surface Protein
NMR	Nuclear Magnetic Resonance
NTS	N-terminal Segment
PAM	Pregnancy-associated Malaria
PCR	Polymerase Chain Reaction

PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PEG	Polyethylenglycol
*Pf*API	*Plasmodium falciparum* Annual Parasite Incidence
PfEMP-1	*Plasmodium falciparum* Erythrocyte Membrane Protein-1
*Pf*PR	*Plasmodium falciparum* Parasite Rate
PoLV	Positions of Limited Variability
RFLP	Restriction Fragment Length Polymorphism
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
TARE	Telomere-associated Repeat Elements
TM	Trans-membrane
TNF-α	Tumor Necrosis Factor-α
TSP	Thrombospondin
Ups	Upstream promoter
VCAM-1	Vascular Cell Adhesion Molecule-1

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1. INTRODUCTION

1.1 A perspective on malaria

Malaria is an infectious disease caused by protozoon parasites of the genus Plasmodium and transmitted by the bite of an infectious female Anopheles mosquito. It is most common in tropical climates, but also occurs in many sub-tropical regions. Malaria infects vertebrates including humans, monkeys, birds, reptiles, and rodents. There are four Plasmodium species causing a clinically relevant infection in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale.* Recently, it has been reported that *Plasmodium knowlesi*, a monkey malaria parasite in certain forest areas of South-East Asia, infects humans more frequently than previously known (Singh et al., 2004).

P. vivax is causing tertian malaria with almost 40% of the world's population at risk. Although often regarded as causing benign and self-limiting infection, there is increasing evidence that overall burden, economic impact and severity of *P. vivax* infection are underestimated (Mendis et al., 2001; Price et al., 2007). According to WHO, there are 132-391 million clinical cases per year. In recent years, increasing numbers of cerebral malaria and fatalities due to *P. vivax* mono-infection have been reported (Tjitra et al., 2008; Tanwar, et al., 2011). *P. ovale* is a more infrequent cause of tertian malaria and often presents with low grade parasitaemia. *P. malariae* infection is also a mild variant of malaria but it can cause renal complications especially in children (Garcia, 2010). *P. falciparum* causing malaria tropica, later abbreviated as "falciparum malaria", causes the most severe clinical symptoms and is potentially lethal in human due to a high reproduction rate and the capability to invade erythrocytes of all ages, thus resulting in very high parasitaemia. Furthermore, infected erythrocytes acquire the ability to adhere to the endothelium of blood vessels causing obstruction of microcirculation, leading to poor perfusion of host tissues, anaerobic metabolism and hypoxia resulting further in multiple organ failure (Miller et al., 2002; Chen[b] et al., 2000).

Malaria cases present with a large range of disease manifestations ranging from asymptomatic infection to severe malaria and death. Some factors are involved in the clinical outcome, including host immunity, parasite virulence and geographical and social factors. Host factors determining the clinical manifestation are age, immune status, proinflammatory cytokines, nutrition and genetic background such as sickle cell trait, thalassaemia and ovalocytosis. The known parasite factors involved are drug resistance, multiplication rate, invasion pathways, cytoadherence, antigenic variation and malaria

toxin. Geographical and social factors such as transmission intensity and population of circulating parasite, access to treatment, cultural and economic factors and political stability also affect the disease outcome (Greenwood et al., 1991; Miller et al., 2002).

Malaria symptoms appear seven days or more after the infective mosquito bite (minimal incubation period). The primary symptom all types of malaria is the 'malaria ague' (chills and fever). Symptoms for uncomplicated malaria are non-specific acute febrile illness, difficult to differentiate from other febrile diseases. Falciparum malaria in non-immune patients, if not treated timely, can progress rapidly to severe illness often leading to death. In highly endemic areas, mostly infants and young children are affected by severe and fatal malaria since semi-immunity develops and protects most of the elder children and adults from severe disease. It is estimated that 0.1-0.5% of all falciparum malaria infections result in severe life-threatening disease (Greenwood et al., 1991). This is only a small proportion, but the overall number of malaria infections is huge enough to result in many cases with life-threatening infection (Snow et al., 2005).

In severe malaria one or more of the following symptoms frequently develop: severe anaemia, prostration, convulsion and respiratory distress, metabolic acidosis or cerebral malaria with impaired consciousness and coma (Mackintosh et al., 2004; WHO, 2000). Early diagnosis and treatment can reduce disease severity, prevent deaths and contribute to the reduction of malaria transmission. Widespread implementation of malaria case-management using effective anti-malarial drugs such as artemisinin-based combination therapy (ACT) and preventive tools such as insectiside-treated bednets, reduced all childhood deaths in Africa by near 20% (Lengeler, 2004). However, resistance to malaria treatment has spread very rapidly, subverting malaria control efforts. Intensive research efforts to develop an efficacious malaria vaccine have been made over the past 20 years but remained elusive because of the complexity of the parasite.

1.2 *Plasmodium knowlesi*

Plasmodium knowlesi is defined as a zoonotic malaria parasite and transmitted by mosquitoes of the *Anopheles leucosphyrus* group. At present, the natural hosts of *P. knowlesi* have been identified as long-tailed macaques (*Macaca fascicularis*), pig-tailed macaques (*Macaca nemestrina*) and leaf monkeys (*Presbytis melalophos*) (Eyles et al., 1962). In 2004, a large number of *P. knowlesi* infections in humans was reported from Malaysia (Singh et al., 2004). Other observations and studies followed recently, including reports

about human fatalities with *P. knowlesi* (Cox-Singh[a] et al., 2008) and cases from Thailand (Jongwutiwes et al., 2004), China (Zhu et al., 2006), Singapore (Ng et al., 2008), the Philippines (Luchavez et al., 2008) and imported by tourists (USA, Finland, Sweden) who had traveled to South-East Asian countries. These findings raised a legitimacy to claim *P. knowlesi* as the fifth causative agent of malaria in humans.

Humans are susceptible to infections and can be infected anywhere within the range of distribution of the specific vector if infected monkeys are present (Collins and Barnwell, 2009). The range of the *A. leucosphyrus* group overlaps with the long-tailed and pig-tailed macaques. The known distributions of *A. leucosphyrus* group are: Southern Thailand, Malaysia, Indonesia, Philippines (Manguin et al., 2008). They are also found in Bangladesh, China, India, Myanmar, and Sri Lanka (Sallum et al., 2005). Long-tailed macaques are widespread throughout the islands of Southeast Asia and mainland Asia (Fittinghoff and Lindburg, 1980; Groves, 2001). Pig-tailed macaques have a wide range throughout Southeast Asia as well (Groves, 2001; Choudhury, 2003). Therefore, naturally acquired human *P. knowlesi* infections have been acquired within this range (Figure 1.1) (Cox-Singh[b] and Singh, 2008). Regarding the large distribution of the vector (*A. leucosphyrus* group) and the natural host of *P. knowlesi* (Macaca species) in Southeast Asia, it was very likely to find *P. knowlesi* in Indonesia.

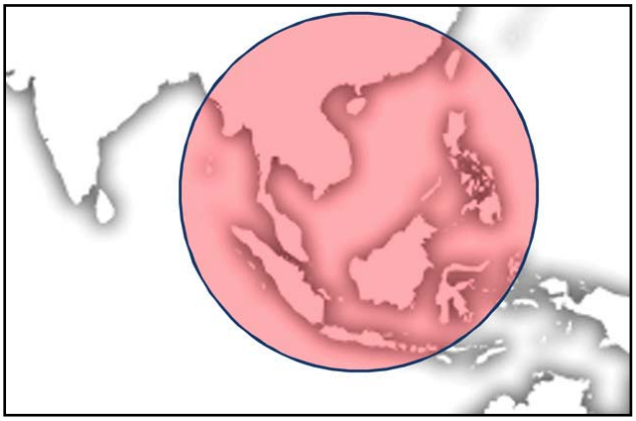


Figure 1.1. Zone where *P. knowlesi* malaria and the anopheline vectors may likely cause infection in humans. *P. knowlesi* is potentially distributed as far north as Taiwan, east as Sulawesi, south to Java in Indonesian archipelago and as far west as Myanmar and the eastern edge of India (Baird, 2009).

The prevalence of naturally acquired knowlesi malaria in humans may be underestimated (Jongwutiwes et al., 2004). Misdiagnosis often occurs by microscopy because the early blood stages of *P. knowlesi* morphologically resemble *P. falciparum*, the mature trophozoites and schizonts may present characteristics of *P. malariae*. Therefore, a

patient infected by *P. knowlesi* is most likely, but not exclusively, to be diagnosed as infected by *P. malariae* (White, 2008).

Several studies in describing the clinical feature of human *P. knowlesi* infection by human volunteers challenged with *P. knowlesi* showed the infection as mild to severe malaria with most attacks terminating spontaneously after two weeks (Coatney et al., 1971). Another study reported initial fever up to 39°C but later fever raised up to 41°C, which appeared daily (quotidian fever) until 10 days and finally diminished spontaneously. Most cases had less than 1% parasitaemia, but one patient showed 12% parasitaemia (Milam and Kusch, 1938). Of interest, African people seemed to be difficult to infect with *P. knowlesi*, four of six challenged developed only very mild disease and the other two none at all. *P. knowlesi* shares very similar Duffy-like receptors on erythrocytes as occurs in *P. vivax*, thus the Duffy receptor negative phenotype probably protects humans against infection by *P. knowlesi* (Chin et al., 1968; Baird, 2009).

1.3 The global distribution of *Plasmodium falciparum* malaria

Malaria is found in 108 countries of the world (WHO, 2010) and every year 500 million cases of malaria and an estimated 1-3 million deaths is reported. In 2007, 2.37 billion people lived in areas at any risk of *P. falciparum* transmission worldwide, 26% located in Africa, 62% in South east Asian and Western Pacific region and the rest is located in American, eastern Mediterranean and European regions (Guerra et al., 2008). Most malaria cases and deaths occur in sub-Saharan Africa (Figure 1.2).

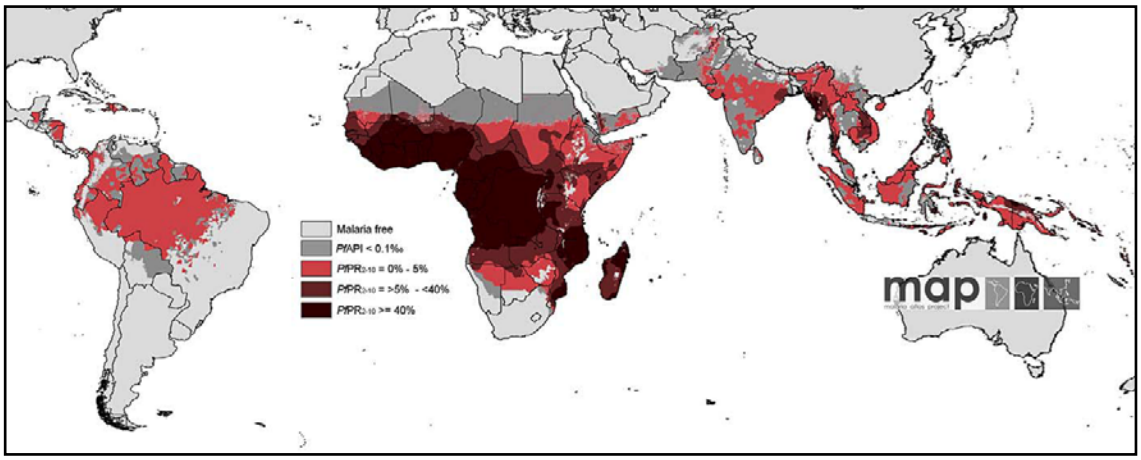


Figure 1.2. The spatial distribution of *P. falciparum* malaria PfPR$_{2\text{-}10}$ prediction stratified by endemicity class. They are categorized as low risk (PfPR$_{2\text{-}10}$ $\leq$5%), intermediate risk (5%<PfPR$_{2\text{-}10}$<40%) and high risk (PfPR$_{2\text{-}10}$ $\geq$40%) areas. The rest area is classified as unstable risk (medium grey area if PfAPI<0.1 per 1,000 pa) or no risk (light grey, if PfAPI=0 per 1,000 pa) (Hay et al., 2009).

In Indonesia, 132.8 million of 230 million people live in malaria endemic areas in 2010. Of these, 70.3% lived in areas of unstable transmission. Among those living in stable transmission, 93.39% were at low risk, 6.6% at intermediate risk and only 0.01% at high risk of transmission (Elyazar et al., 2011). In 2009, 544 thousands confirmed malaria cases with 900 deaths were reported according to WHO (SEARO-WHO, 2010).

The intensity of malaria transmission depends on factors related to the parasite, the vector, the human host, and the environment. There are approximately 33 different *Anopheles* vector species around the world. Some species are more significant than others as vectors because of variations in susceptibility to the parasite or the propensity to bite humans (Kiszewski et al., 2004). Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Immunity is developed over years of exposure. Though it rarely results in sufficient protection, meaning asymptomatic parasitaemia, like in high transmission areas, frequent exposure does decrease the risk of severe malaria infection. For the children until the age of 6 months, they develop only mild symptoms and low parasitaemia (Snow et al., 1997) as the result of passive transfer of maternal antibodies and persistence of haemoglobin F (Sehgal et al., 1989; Hogh et al., 1995). From around 6 months, children become susceptible to severe disease and death. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with lower transmission and less immunity, all age groups are at risk.

Transmission also depends on climatic and ecological factors. These factors are required for the abundance and survival of the anopheline mosquitoes and for the development of sporozoites within the vector. Beside the climatic conditions, transmission can be influenced by human activities such as urbanization, mass population movement, agricultures and malaria control program.

1.4 *Plasmodium falciparum* life cycle

The life cycle of *P. falciparum* includes sexual and asexual reproduction, involving both human and *Anopheles spp* mosquito hosts. During the entire cycle, there are several changes (Figure 1.3). When an infected female *Anopheles* mosquito injects saliva during blood meal to prevent coagulation, it inoculates 1-100 infective sporozoites into the blood stream, infecting the human (Rosenberg et al., 1990; Ponnudurai et al., 1991). The blood and probably the lymphatic system transport the sporozoites into the liver where

5-10% successfully invade hepatocytes (Ferreira et al., 1986). They differentiate into hepatic schizont in which around 30,000 daughter merozoites develop (Amino, 2006). After 5-14 days the schizont ruptures and spreads thousands of merozoites into the blood stream, they invade erythrocytes. The development from sporozoite injection to hepatocyte rupture is called the exo-erythrocytic cycle. During this stage, the host stays asymptomatic.

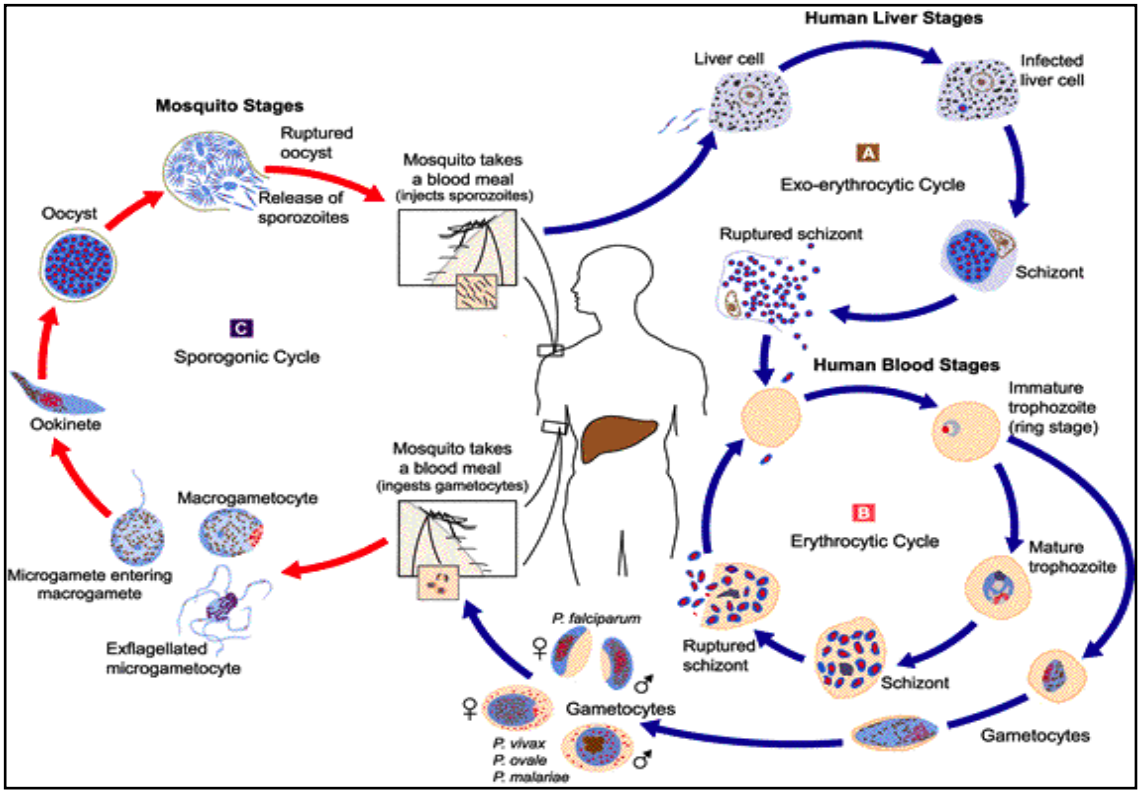


Figure 1.3. Schematic representation of *P. falciparum* life cycle. The different phase of *P. falciparum* life cycle, (A) the exo-erythrocytic cycle occurs in the hepatocytes from sporozoite injection to hepatocyte rupture; (B) asexual reproduction in erythrocytes and differentiation into gametocytes in the erythrocyte cycle; (C) the sexual reproduction in the mosquito's mid gut and the completion of the life cycle by injection of sporozoites into the new host (CDC, www.dpd.cdc.gov/dpdx).

The asexual reproduction begins in the erythrocytes. After invasion, the merozoite grows and develops into a small ringform. The cells are in the G-phase and increase in size, called trophozoite stage. The transition into the S-phase with DNA duplication and membrane separation leads to the schizont form, containing around 16-32 merozoites. The infected erythrocyte (IE) ruptures and releases these daughter merozoites into the blood stream where they each invade a fresh erythrocyte and start the new cycle. The entire cycle from invasion to rupture of the erythrocyte takes around 48 hours. Once these parasites begin affecting erythrocytes, the disease becomes clinically apparent. It is

thought that the intermittent release of merozoites causes the symptoms of malaria in the host by stimulation of endogenous release of pyrogens. The majority of merozoites will again infect erythrocytes and develop into schizonts. A small number will differentiate into male and female gametocytes within erythrocytes. These sexual forms are responsible for transmission of *P. falciparum* through ingestion by feeding mosquitoes.

When a female anopheline mosquito feeds on the blood of an infected person, it ingests gametocytes. Inside the mosquito's midgut, the female gametocytes develop into macrogametes and the male gametocytes exflagellate and form microgametes which fertilize the macrogametes by fusion, forming motile zygotes called ookinetes. The diploid ookinetes cross the midgut membrane, undergo meiosis and adhere onto the exterior site of the gut wall. Here, they undergo several rounds of mitosis to form oocyst (sporogony). This step is called sporogonic cycle. Each oocyst releases thousands of motile haploid sporozoites into the mosquito's body cavity, from where they migrate into the mosquito's salivary glands. During the next blood meal, they are injected together with the saliva into a new host and the cycle is completed.

1.5 *Plasmodium falciparum* erythrocyte membrane protein–1 (PfEMP-1)

During the erythrocytic cycle, *P. falciparum* expresses a large and highly polymorphic *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), which is exported from the parasite to the surface of infected erythrocyte approximately 18 hours post invasion onwards. This protein has been linked to two key phenomena responsible for the pathology associated with *P. falciparum* infection: cytoadherence of IE and immunoregulatory effects on host immune cells through binding to other host receptor and antigenic variation with consequent immune evasion.

PfEMP-1 is ~250 – 350 kDa in size and varies in domain composition and binding specificity. The architecture of PfEMP-1 is complex. The extracellular part protrudes from the erythrocyte membrane into the host's blood plasma being the most variable part of the protein. It contains an N-terminal segment (NTS) followed by a segment composed of three binding domain types: Duffy binding-like (DBL) domain, cysteine-rich interdomain regions (CIDR) and C2 domain. The NTS is semi-conserved and located at the N-terminal end of PfEMP-1. It contains sequence features sufficient for transport beyond the parasitophorous vacuole that surrounds the intraerythrocytic parasite (Hiller et al., 2004; Marti et al., 2004). The DBL domain is an adhesive region that binds a variety of different receptors. Based on conserved sequence stretches and conserved cysteine in the sequences, the DBL domain is classified into six different types: α, β, γ, δ, ε and χ.

Further sequence analysis revealed that each DBL domain is divided into 10 semi-conserved blocks (A-J) and 10 variable blocks (1-10). The semi-conserved blocks contain a higher number of invariant or bio-chemically-conserved residues that are presumably important for domain folding. The absolute number of cysteine residues differs significantly between DBL domains of PfEMP-1. The CIDR domain consists of semi-conserved stretch and is classified into three different types: α, β and γ. The last domain type C2 involved in calcium-dependent phospholipid binding and membrane targeting process such as sub-cellular localization (Smith[b] et al., 2000). The intracellular part or the acidic terminal sequence (ATS) is rather conserved and may function as an anchor by interaction with erythrocyte skeleton proteins (Oh et al., 2000) and additional parasite proteins such as KAHRP (Rug M et al., 2006; Waller et al., 2002).

The PfEMP-1 proteins have related protein architecture, but the sequence, number, location and type of domains differ significantly, usually they contain between two and seven DBL domains and one and two CIDR domains (Figure 1.4). Thirty-one different architectural types are described from the three sequenced parasite *var* repertoires (Kraemer et al., 2007). Because the domain architecture is variable, PfEMP-1 domains are identified by position in the protein and by type, for example DBL1α represents the first domain after the NTS. Semi-conserved head structures like DBL1α–CIDR1α, DBL2β-C2 and DBLδ–CIDRβ make up a protein prototype and are found in nearly all PfEMP-1 molecules. By integration of additional domains, the flexibility of protein may increase and provides an extra advantage, such as the ability to bind multiple receptors (Smith[b] et al., 2000).

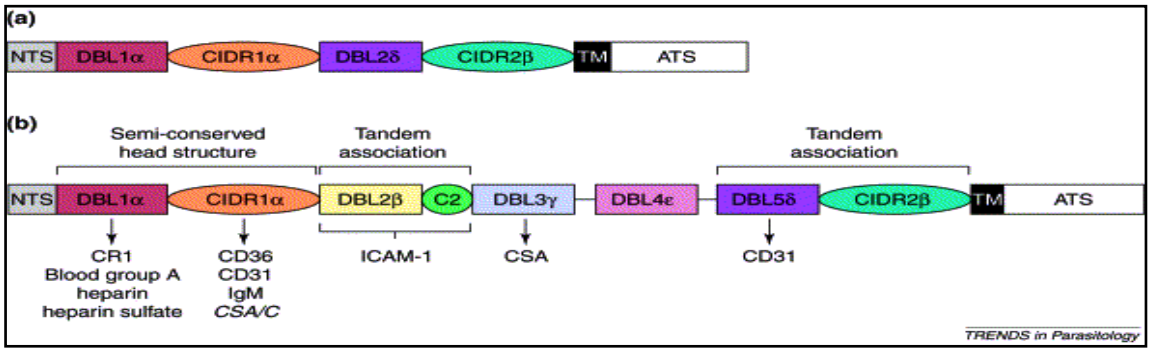


Figure 1.4. Schematic architecture of PfEMP-1. (a) A small PfEMP-1 protein consists of minimal arrangement i.e. the NTS domain followed by the semi-conserved DBL1α-CIDR1α and the ATS domain. (b) A larger variant of PfEMP-1 including the semi-conserved head structure DBL1α-CIDR1α, tandem association DBL2β-C2 and DBL5δ-CIDR2β and additional DBL and CIDR domain. The host cell receptors involved in binding are indicated at the respective PfEMP-1 binding-domain (Smith et al., 2001).

1.6 *var* gene family

PfEMP-1 is encoded by the highly divers *var* gene family consisting of approximately 60 genes per haploid genome. The *var* genes have two exons with a ~170 bp to 1.2 kb intron. The first exon is highly polymorphic, encodes the extracellular part of protein and trans-membrane (TM) domain and varies in size between 3.5 and 9.0 kb. The second exon, in contrast, is relatively short, more conserved and encodes the intracellular acidic terminal segment and the size is between 1.0 and 1.3 kb (Figure 1.5b) (Gardner et al., 2002).

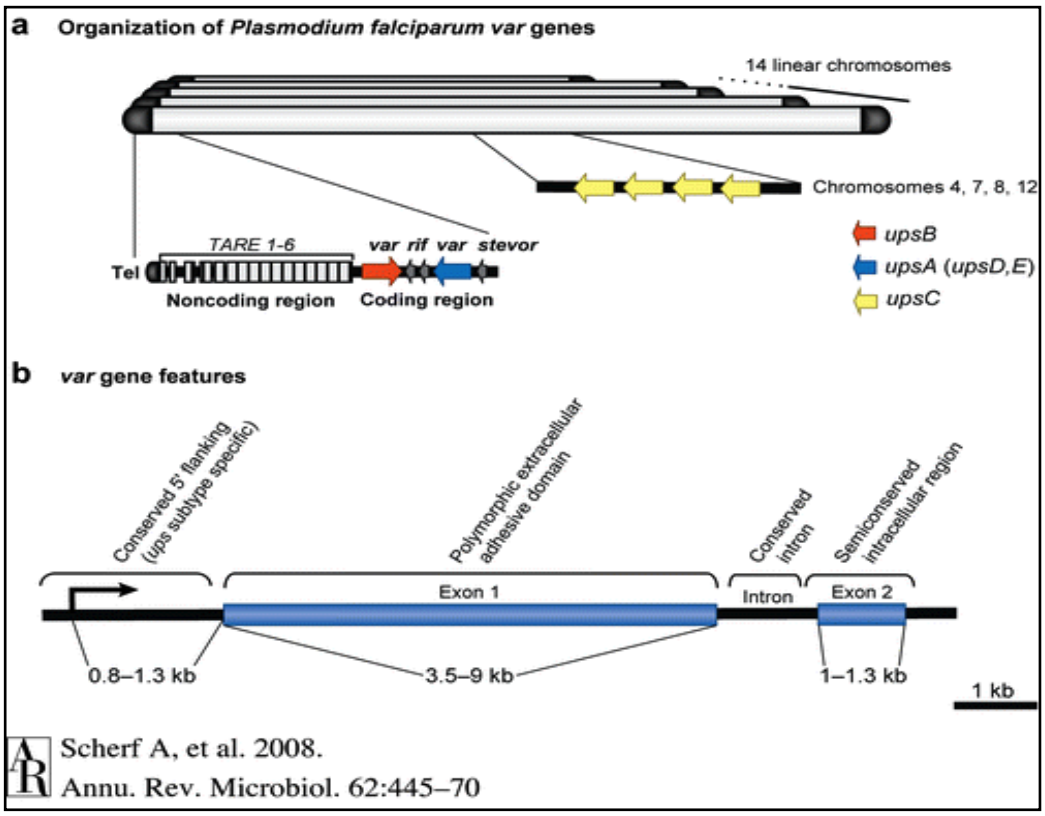


Figure 1.5. Genomic organization and the features of *var* genes. (a) All 14 *P. falciparum* chromosomes have a common overall organization in sub-telomeric regions, with non-coding repeats of variable size called TARE (telomere-associated repeat elements), 1-6 located next to telomere repeats. The region is followed by a member of *var* gene family with the second *var* gene transcribed in the opposite direction. *rif* gene are often located between *var* genes. Several tandem arrayed *var* gene clusters are also found in the centromere. *var* genes classification based on the upstream promoter type (Ups) and chromosomal location, arrows indicate the direction of transcription. UpsB, UpsA and UpsE located at sub-telomere but have opposite transcriptional direction; Ups C located at central chromosomal region with the transcriptional direction toward centromere. (b) A common feature of the *var* gene family. All members contain two exons separated by conserved intron. Exon 1 encodes for the highlypolymorphic extracellular domain and Exon 2 is more conserved and encodes the intracellular region (Scherf et al., 2008).

In 3D7, *var* genes present on all 14 chromosomes and approximately 60% of *var* members are located at sub-telomeric regions, with the remainder in chromosome central regions

(Gardner et al., 2002). One to three *var* genes exist at telomeres pointing in different direction, either in tail-to-tail (most frequent), head-to-tail, or head-to-head orientation relative to each other with one or more *rif* gene in between. The central *var* genes appear single or in groups that are nearly always tandem arrayed in the head-to-tail orientation, containing three to seven *var* genes (Figure 1.5a) (Kyes et al., 2007).

The chromosomal location and orientation of *var* genes are associated with particular types of 5'-flanking regions called Ups. Based on the 5'-Ups type, domain structure of the encoded protein and sequence similarities in coding and non-coding regions, *var* genes can be classified into three major group: A, B and C (Gardner et al., 2002; Lavstsen et al., 2003). UpsA type is located sub-telomeric and transcribed towards the telomere. UpsB type is telomeric and transcribed towards the centromere, while UpsC type is located in the center of chromosome (Figure 1.5a). There is also a subset of *var* genes, the B/C group, which has UpsB-like promoters but present in central chromosomal regions and B/A group which present in sub-telomeric chromosomal region but have gene orientation to centromere (Lavstsen et al., 2003). This general gene organisation is retained across the parasite isolate; it has been postulated to influence the functional and antigenic specialization of PfEMP-1 proteins through gene recombination hierarchies (Kraemer and Smith, 2003).

Apart from *var* groups A, B, and C, there are three unusual semi-conserved *var* genes; *var*1CSA, *var*2CSA and type 3 *var* gene. According to their chromosomal location and transcriptional direction, *var*1CSA and *var*2CSA belong to *var* group A, however both have a distinct 5'-Ups, *var*1CSA has an UpsD, which is now grouped with UpsA (Kraemer et al., 2007) and *var*2CSA has an UpsE. And type 3 *var* has UpsA. All three genes are exceptionally conserved across parasite isolates and constitute possible gene orthologs but they share little sequence identity with other members of the *var* gene family, posing the hypothesis that these genes primarily undergo self-self recombination (Kraemer and Smith, 2006).

1.7 *var* gene diversity

Since the *var* gene repertoire is around 60 copies per haploid genome, it is not surprising that large sequence diversity exists between different isolates (Su et al., 1995). A study using *in situ*-hybridization techniques found that genetically diverse parasites contained essentially, although not completely, non-overlapping *var* gene repertoires, indicating the extreme diversity of *var* genes (Freitas-Junior et al., 2000). Global sequence

comparisons have also demonstrated the immensity of *var* gene diversity with nearly no overlaps among repertoires from different geographical origins (Barry et al., 2007) nor between isolates (Kaestli et al., 2004; Bull et al., 2005). However, the mechanisms driving *var* gene diversity remain only partially understood. Genetic diversity can be generated by recombination and rearrangement during the sexual (meiotic) (Taylor[b] et al., 2000) or asexual (mitotic) phase of the parasites life cycle (Duffy et al., 2009). A study analyzing progeny clones from two genetic crosses supported the high rate of recombination in *var* genes leading to a new *var* forms via gene conversion involving the nuclear structure as the hot spots of recombination (Scherf et al., 2008). Another approach reported that sub-telomeric *var* genes are in part responsible for *var* gene diversity by mechanism of intragenic recombination and re-arrangement between two *var* genes (Taylor[b] et al., 2000). Recombination preferentially occurs within genes which have a common genome location and gene orientation, and is likely influenced by 5'-flanking regions and gene coding similarity (Kyes et al., 2007). This recombination hierarchy may be shaping the *var* gene repertoire and influencing the evolution of the gene family.

1.8 Regulation of *var* gene expression

Several important findings regarding *var* gene expression have been reported. First, expression of *var* genes is stage specific and temporally regulated during parasite development (Kyes et al., 2000). Second, telomeric as well as centromeric *var* genes located on different chromosomes can be selected for expression. Third, the expression of *var* genes is mutually exclusive, i.e. only one member of the *var* gene family is expressed on the surface of an infected erythrocyte (Chen et al., 1998; Scherf et al., 1998).
The expression of PfEMP-1 is developmentally regulated as the protein appears on the surface of the infected erythrocyte around 16-18 hours post invasion (Gardner et al., 1996). The control of *var* gene transcription is stage-specific and tightly regulated. Two opposing results from two different approaches were reported. Northern blot analysis showed that the major *var* mRNA transcript encoding the PfEMP-1 was present at maximum amount in late-ring stage (3-18 h post-erythrocyte invasion) declining until barely detectable over the following 6 h by trophozoite stage, suggesting that the control of *var* gene expression is maintained at the level of transcriptional initiation (Kyes et al., 2000). In contrast were results from reverse transcription-PCR (RT-PCR) experiments detecting mRNA transcripts within a single IE. Multiple *var* transcripts were present in ring stages with one dominant transcript present in the trophozoite stage which implies a post-transcriptional mechanism of control (Chen et al., 1998; Scherf et al., 1998). Of

interest, a repression of *var* gene transcription during intra-erythrocytic development of the parasite occurs at different times depending on the chromosomal location of the *var* gene. Sub-telomeric *var* genes are only expressed up to 18 hours post infection and inhibition of sub-telomeric *var* gene transcription occurs between 4 and 8 hours before transcription of centrally located *var* genes. This indicates that the structural differences of the promoter types reflect functional and regulatory differences in transcriptional repression (Voss et al., 2003).

var gene expression follows the rule of mutually exclusive expression, with only one gene being expressed by an individual parasite at a given time (Scherf et al., 1998). The mutually exclusive expression is not based on a negative feedback from the *var* protein product itself but regulated at the level of transcription initiation level (Kyes et al., 2007) and involves multiple layers of control including the epigenetic factors.

The first layer is the structure of the two transcriptionally active promoters of *var* genes; one upstream of the first exon (5' *var* promoter) encoding mRNA and the second within an intron (*var* intron promoter) that leads to expression of sterile (non-coding) RNA and has regulatory functions both as a silencer and in the recognition of *var* genes (Deitsch et al., 2001; Frank et al., 2006; Dzikowski et al., 2007). The interaction between these two promoters and the presence of sterile RNA (Calderwood et al., 2003; Kyes et al., 2003) seems to play an important role in the silencing process of *var* genes.

The second layer is the epigenetic memory of *var* genes, i.e. once the transcriptional status of an individual *var* gene has been established, the state (either active or silent) tends to be maintained through multiple cell cycles with only rare switching (Horrocks et al., 2004; Frank et al., 2007). Thus, the transcriptional state of each gene is 'remembered' from one cell cycle to the next and this memory is encoded into the genome through a series of 'marks' placed at each gene. In addition, the particular modification of histone including acetylation and methylation plays a significant role in 'bookmarking' either active or silent genes (Duraising et al., 2005, Freitas-Junior et al., 2005; Comeaux and Duraising, 2007).

The third layer is the sub-nuclear organization, where the silent genes tend to localize within regions of the nucleus that contain primarily condensed heterochromatin and active transcription commonly takes place in euchromatic regions in which chromatin is loose and open for transcription. Study using *var*-specific probes showed that all *var* genes localize mostly at the nuclear periphery regardless of chromosomal location or their activation state and *var* genes appear to move upon changes in their transcriptional

activity. A model was proposed that the telomeric clusters are located within heterochromatin region of the nuclear periphery whereas upon activation it moves to another location of the nuclear periphery where the chromatin is open for transcription (Ralph et al., 2005).

1.9 Antigenic variation and *var* gene switching

P. falciparum is able to avoid clearance by the human immune system which depends on its capacity to continuously alter the surface exposed antigenic proteins which are vulnerable to antibody recognition and attack, this process is called antigenic variation (Dzikowski and Deitsch, 2009). The antigenic variation mechanism is carried out by successive switching of expression of *var* gene family members (Horrocks et al., 2004). This allows parasites to modify the antigenic and functional properties of IE resulting in modified adhesive phenotypes and possibly altered virulence. Moreover, antigenic variation allows parasites to establish persistent chronic infections.

In vitro observation has shown that the rate of switching is an intrinsic property of each *var* gene, different *var* genes switch on and off at different rates, and no preferential switching between central or telomeric *var* gene occurs (Horrocks et al., 2004). However, another study suggested that switching relies on an epigenetic process and correlates with chromosomal location, and that in clonal parasite populations, central *var* genes have extremely stable expression pattern and rarely undergo transcriptional switches in the absence of selection. Sub-telomeric *var* genes on the other hand readily switched to alternative *var* loci (Frank et al., 2007).

Parasites cultured *in vitro* switched spontaneously at a rate of 2% in the absence of immune pressure (Roberts et al., 1992). If the *in vivo* switching rate would occur to the same degree, the parasite would exhaust its variant repertoire, instead of new repertoires of variant antigens becoming available for selection through recombination events. Nevertheless, by combining new data derived from naive human hosts with a simulation model, data suggest that during the acute stage of infection, switching rates per generation is ~18%, this is even much faster than initially thought (Gatton et al., 2003), so it is likely that switching rate has a wide range.

The signals controlling the switching of expression within the parasite are unclearly defined. Some evidences suggest that there is a communication between the host and the parasite and the homologous anti-variant antibody acts as a signal from host to parasite to induce switching (Brown, 1973).

1.10 Cytoadherence and its consequence on clinical manifestation

P. falciparum is able to modify both of the membrane, cytoplasma and the surface of the infected erythrocytes (IE). By these modifications, the parasite is enabled to adhere to other host cells during trophozoite and schizont stages, called cytoadherence (Moxon et al., 2011). Three major types of cytoadherence have been described: sequestration if the IE adhere to endothelial cells (Udeinya et al., 1981), rosetting if IE bind to uninfected erythrocytes (Udomsangpetch et al., 1989) and auto-agglutination or clumping if the activated platelets bind with IE, forming a large cluster of cells (Pain et al., 2001).

Sequestration

P. falciparum-infected erythrocytes are found to be sequestered in various organs including brain, heart, lung, liver, kidney, sub-cutaneous tissues and placenta (Miller, 1969; Yamada et al., 1989). Through successive cycles of sequestration and multiplication, parasites reach sufficient densities in micro-vascular beds to cause both organ specific and systemic disease. While sequestration is essential for the parasite's survival by evasion of the spleen clearance mechanisms, it has severe consequences for the host. Parasite's sequestration will occlude the capillary blood vessels, leading to hypoxia, metabolic acidosis and dysfunction of affected organs. Massive sequestration in the brain is believed as the underlying cause of coma in cerebral malaria, involving the role of host receptor intercellular adhesion molecule-1 (ICAM-1) (Chen et al., 2000). In addition, binding of IE to syncytiotrophoblasts resulting in placental sequestration occurs in pregnancy-associated malaria (PAM).

Rosetting

Another type of cytoadherence, the adhesion of IE to uninfected erythrocytes, is called rosetting. Rosetting has been observed in the peripheral blood of patients with acute severe malaria and in blood vessels during autopsy of malaria victims (Riganti et al., 1990; Ho et al., 1991; Scholander et al., 1996). Some studies with largely different geographical and epidemiological setting also reported an association between rosetting forming capacity and disease severity (Carlson et al., 1990; Treutiger et al., 1992; Rowe et al., 1995; Udomsangpetch et al., 1996; Newbold at al., 1997; Kun et al., 1998; Heddini et al., 2001). Others reported that antibodies which disrupt rosettes are less common in individuals with clinical disease (Carlson et al., 1990; Reeder et al., 1997) and serum from severe malaria patients usually contains a low titer of anti-rosette antibodies, while

patients with uncomplicated malaria have a higher titer of anti-rosette antibodies (Barragan et al., 1998; Treutiger et al., 1992).

Rosetting has a masking effect for IE. As the IE is in the middle and completely covered by uninfected erythrocytes, no antibodies or immune systems can recognize and eliminate the parasites. The aggregation of erythrocytes even enhances the negative effect of sequestration, because constricted capillaries due to sequestered IE, may be completely blocked by floating rosettes. In post-capillary venules, IE adhered to the endothelium and the uninfected erythrocytes formed rosettes on top of the adherent cells (Kaul et al., 1991), suggesting linked similar phenotypes between rosetting and sequestration. Some erythrocyte-rosetting receptors are also expressed on the endothelial cells, indicating a dual role in endothelial cytoadherence and rosetting (Vogt et al., 2003). This phenomenon is observed in the peripheral blood of patients with severe malaria and is thought to be an underlying cause of coma (Scholander et al., 1996).

Auto-agglutination and clumping

The ability of IE to adhere to neighbouring IE and thereby forming large aggregates without involving any uninfected erythrocytes is termed as auto-agglutination. This phenomenon is common in natural infections and a correlation with the disease severity has been shown (Roberts et al., 2000). Clumping is another adhesive phenomenon presented by IE, it is mediated by platelets and involved CD36 as a receptor adjoining IE to form large clumps (Wahlgren et al., 1995; Pain et al., 2001). Platelets serve as a bridge between CD36 and the surface of endothelial cells, providing receptors for adhesion to microvasular beds that otherwise lack adhesion receptors (Wassmer et al., 2004). However, not all CD36 binding isolates show this phenotype, indicating the involvement of other receptors. A platelet-mediated clumping varies between parasite isolates. Some studies reported a significant association of clumping with severe malaria (Pain et al., 2001; Chotivanich et al., 2004; Wassmer et al., 2008).

1.11 Host receptors involved in cytoadherence

The affinity of *P. falciparum* for binding to endothelial cell receptors is diverse, as its role in sequestration and also depends on the host receptor specificity. Infected erythrocytes are able to bind to a range of host receptors with each parasite having the ability to bind a subset of them only.

Numerous host receptors mediating binding to the IE have been identified. CD36 (Barnwell et al., 1985; Ockenhouse et al., 1989), thrombospondin (TSP) (Roberts et al.,

1985), intercellular adhesion molecule-1 (ICAM-1) (Berendt et al., 1989), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Treutiger et al., 1997), vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule (ELAM) (Ockenhouse et al., 1992), E-selectin (Ockenhouse et al., 1992) and P-selectin (Udomsangpetch et al., 1997) are involved as receptors in the binding to endothelial cells. Chondroitin sulfate A (CSA) (Robert et al., 1995; Rogerson et al., 1995), hyaluronic acid (HA) (Beeson et al., 2000) and IgG (Flick et al., 2001) are the main receptors involved in sequestration in placental malaria.

ICAM-1

ICAM-1 is an 80-114 kDa glycosylated variabel membrane glycoprotein consisting of five Ig-like domains. It belongs to the immunoglobulin superfamily and is expressed on the surface of a wide range of cell types including endothelial cells and leukocytes (van de Stolpe and van der Saag, 1996; Ho et al., 2000). ICAM-1 is widely expressed on cerebral endothelium and leukocytes, and up-regulated by pro-inflammatory cytokines such as tumor necrosis factor (TNF-α). ICAM-1 has implicated to play a role in severe disease because the adhesion to ICAM-1 tended to be higher in patients with cerebral malaria (Newbold et al., 1997). In addition, there was a co-localization of ICAM-1 with parasite sequestration in brain vessels in autopsy samples from cerebral malaria patients (Turner et al., 1994). Further, up-regulation of ICAM-1 expression on endothelium during malaria infection was observed (Turner et al., 1998).

A high frequency mutation in ICAM-1, called ICAM-1^{kilifi}, was present in individuals from East and West Africa but absent in Europeans (Fernandez-Reyes et al., 1997). The studies on the effect of this mutation on susceptibility to severe malaria showed conflicting results. A study in Kenyan children found that homozygotes for this mutation were found to be at a two-fold higher risk of cerebral malaria than controls (Fernandez-Reyes et al., 1997), but another study in West Africa (Gambia and Gabon) reported the opposite association (Bellamy et al., 1998; Kun et al., 1999).

Using a genome-wide approach, it was shown that only some PfEMP-1 variants containing the DBLβ-C2 domain are able to bind to ICAM-1 (Howell et al., 2008). The low affinity of most malaria parasites for this receptor indicates that ICAM-1 only mediates IE rolling on the endothelial lining; stable binding most likely occurs synergistically with other receptors such as CD36 (McCormic et al., 1997).

CD36

CD36 is an 88-kDA glycoprotein scavenger receptor found on the surface of various cells including platelets, macrophages, monocytes, leukocytes, dendritic cells, epithelial cells and microvascular endothelial cells (Greenwalt et al., 1992). CD36 expression on cerebral endothelium of cerebral malaria patients was very little, but there was ubiquitously on lung, liver, kidney, skin and muscle vasculature (Turner et al., 1994; Turner et al., 1998). In contrast with ICAM-1 expression, CD36 and TSP are not sensitive to IFNγ or TNFα stimulation. Binding to CD36 and likewise to TSP seems to be a constitutive feature of most adherent parasite (Ockenhouse et al., 1991; Newbold et al., 1997), although binding to these receptors does not correlate with severity of disease (Turner et al., 1994; Newbold et al., 1997).

Thrombospondin (TSP)

TSP is an adhesive glycoprotein released into plasma in response to platelet activation by thrombin. IE bind to purified TSP in static assays and bind to endothelial cells via TSP under flow conditions (Heddini et al., 2001; Roberts et al., 1985). A study investigating the association of TSP binding with severe malaria found that although most Kenyan field isolates adhered well to TSP in static assays, there was no correlation with disease severity (Heddini et al., 2001).

P-selectin

P-selectin (CD62P) is a glycoprotein that express on activated platelets and endothelial cells and is important for leukocyte trafficking. Studies in Thai field isolates showed that P-selectin mediates rolling of IE on endothelial cells and facilitates adhesion to CD36 (Yipp et al., 2007; Udomsangpetch et al., 1997; Ho et al., 1998).

PECAM-1/CD31

PECAM-1/CD31 is widely expressed on endothelial cells, monocytes, platelets and granulocytes. IE bind to PECAM-1 on endothelial cells, and the binding site has been mapped to the first four immunoglobulin-like domains of PECAM-1. Parasite binding to PECAM-1 is up-regulated by IFN-γ. Although PECAM-1 binding parasites were almost as frequent as CD36-binding parasites in Kenyan isolates and parasites from patients with severe manifestation had a greater tendency to bind PECAM-1, the correlation with malaria severity was not significant (Heddini et al., 2001).

VCAM-1 (CD106)

VCAM-1 is a member of the immunoglobulin superfamily and expressed by cytokine-activated endothelium. Thai field isolates were shown to bind and roll on VCAM-1, but static adhesion did not occur. In African isolates, binding to VCAM-1 was extremely low and not associated with disease severity (Newbold et al., 1997).

E-selectin (CD62E)

E-selectin is a glycoprotein expressed on endothelial cells at sites of inflammation. In African isolates, E-selectin binding was extremely low and not associated with disease severity. The role of E-selectin in cytoadherence is thus probably minor (Newbold et al., 1997).

CSA and HA

CSA and HA are especially well expressed on the surface of syncytiotrophoblast layer of the placenta. They mediate sequestration in pregnancy-associated malaria (PAM) (Beeson et al., 2000; Beeson et al., 1999; Maubert et al., 2000). Further, parasite binding to immunoglobulins may contribute to IE sequestration in the placenta. Immunglobulins act as a bridge between PfEMP-1 and the placenta, thereby assisted by CSA in placental adhesion (Flick, 2001; Rasti et al., 2006). Several studies have shown that parasite isolated from infected placenta typically adhere to CSA and/or HA but not to endothelial receptors such as ICAM-1 and CD36, and they do not form rosettes. Parasites isolated from children or non-pregnant adults, showed little or no adherence to CSA (Beeson et al., 1999).

A number of different erythrocyte surface ligands are described to be involved in rosetting, including the blood group A and B antigens (Barragan et al., 2000), complement receptor 1 (CR1) (Rowe et al., 1997), heparan sulphate (HS) (Rogerson et al., 1994; Rowe et al., 1994; Vogt et al., 2004), CD36 and glycosaminoglycans (GAGs) (Scholander et al., 1996; Chen et al., 1998; Handunnetti, 1989). All receptors are glycosylated, indicating that it is crucial for the interaction.

A and B blood group antigens

The A and B blood group sugars are trisacharides attached to a variety of erythrocyte glycoproteins and glycolipids, and are also found on platelets, leukocytes and

endothelial cells. All isolates tested showed a high preference for either A or B blood group erythrocytes (Udomsangpetch et al., 1993). Further, individual with blood A group antigen phenotype were more likely to suffer severe malarial disease and coma than those of other blood group (Fischer and Boone, 1998). Human genetic studies support a direct role of A- and B-mediated rosetting in the pathogenesis of severe malaria, because blood group O reduces rosetting in field isolates and confers significant protection against severe malaria (Rowe et al., 2007; Fry et al., 2008; Pathirana et al., 2005).

Complement Receptor 1 (CD35)

CR1 is a complement regulatory protein and widely distributed on the surface of erythrocytes, leukocytes and dendritic cells (Khera and Das, 2009). It is an important rosetting receptor, since individual with CR1-deficiency was associated with protection from severe malaria (Cockburn et al., 2004). Some parasites cultured in CR1-deficient erythrocyte lose their capacity to form rosettes. Further, a monoclonal antibody against CR1 reverses rosetting, and deletion mutant of CR1 reverses rosetting in both laboratory and field strains (Rowe et al., 1997; Rowe et al., 2000).

Heparan Sulphate (HS) and Glycosaminoglycan (GAG)

Heparan sulphate (HS) and glycosaminoglycan (GAG) are involved in binding of rosetting IE to endothelial cells (Chen et al., 1998). The rosettes of most wild isolates can be disrupted by GAG, suggesting that malaria parasites use a GAG on the surface of erythrocyte as a rosetting receptor (Rogerson et al., 1994). GAG disrupts rosettes of FCR3S1 parasites and block rosette formation (Barragan et al., 1999). In a Kenyan field-isolate study, binding of fluorescently labeled heparin was significantly higher in isolates from patients with severe malaria than those from uncomplicated disease, supporting a role of HS in severe malaria (Heddini et al., 2001).

1.12 The role of PfEMP-1 in cytoadherence

A lot of receptors have been described to be involved in cytoadherence in human hosts, but only few molecules from parasites have been proposed to serve as ligands for cellular adhesion. The best characterised molecule involved in cytoadherence is the PfEMP-1. PfEMP-1 is deposited on the surface of IE on knob-like structure from approximately 18 hours post invasion onwards. The interaction between PfEMP-1 with a large number of different host receptors requires several binding domains in the PfEMP-

1 protein. The sequencing of *var* genes has enabled the mapping of specific receptor binding sites within PfEMP-1. There are several binding sites for numerous host receptors at the N-terminal of extracellular domain of PfEMP-1.

DBL1α

It is known that *P. falciparum* strain R29 binds CR1 on unifected erythrocytes via DBL1α domain to form rosettes (Rowe et al., 1997; Chen et al., 1998). The DBL1α domain also mediates heparin sulphate binding leading to sequestration. Binding residues for the CR1 lie in the central region of R29-DBL1α between conserved cysteine C5 and C12.

A specific variant from a rosetting parasite clone binds to the blood group A trisacharide via the DBL1α domain to form rosettes (Chen[a] et al., 2000). Although the majority of *var* genes appear to have a DBL1α domain, not all parasite isolates form rosettes or adhere to the receptors involved. It is important to stress that not all expressed *var* genes have the same binding properties and domain-specific binding varies considerably between *var* genes.

CIDR1α

CIDR1α is the domain mediating binding to CD36. Binding to CD36 is interesting as it is a feature of many parasite isolates (Ockenhouse et al., 1991, Newbold et al., 1997). Sequence analysis from diverse parasites corresponding to the CD36 binding region showed that CIDR1α sequences were not identical but displayed some homology, presented by a conservation of cysteine residues at the end of the fragment (Baruch et al., 1997).

An exception to CIDR1α-mediating binding to CD36 is an *in vitro*-selected CSA-binding parasite isolate, which revealed CSA-binding through a CIDR1α domain. As a consequence of binding to CSA through the same CIDR1α domain, CSA and CD36-binding are mutually exclusive phenotypes (Gamain et al., 2002). In addition, CIDR1α together with DBL2δ domains of specific PfEMP-1 variants are thought to have binding capability to PECAM-1 (Chen[a] et al., 2000).

DBLβ-C2

DBLβ-C2 is a PfEMP-1 domain mediating binding to ICAM-1. Binding residues for ICAM-1 lie in the central and terminal region of DBLβ-C2 domain between conserved cysteine C3 and C16 for ICAM-1 binding isolates ((Smith[a] et al., 2000; Mayor et al., 2005;

Springer et al., 2004). Alignment of ICAM-1 binding DBLβ-C2 domains from three isolates (A4, A4tres and JDP8) revealed significant homology sharing 16 conserved cysteine residues and a number of conserved hydrophobic amino acid residues, indicating conservation of the structure as well as three-dimensional structure. Furthermore, multiple sequence alignments of ICAM-1 binding and non binding DBL2β-C2 domains suggested that the binding region for ICAM-1 is unlikely to lie in a linear sequence stretch within DBL2β-C2 (Chattopadhyay et al., 2004).

DBLγ

Although DBLγ domain has been linked to CSA binding, many expressed DBLγ domains do not bind CSA. One of the DBLγ domain binding CSA is expressed by the FCR3*var*CSA gene (Buffet et al., 1999). The binding residues for CSA from FCR3*var*CSA lie in the central region of DBLγ between cysteines 5 and 8 (Mayor et al., 2005). But, among those domains that bind CSA there are varying amounts both of homology and diversity in sequence without any clear conserved areas (Gamain et al., 2004). Of interest, *var2CSA* gene which is dominantly transcribed in many placental isolates and CSA-selected isolates, has an atypical structure lacking both DBLγ and CIDR1α domains instead possessing three DBLχ-type and three DBLε-type domains (Salanti et al., 2003). However, the role of *var*2CSA in CSA binding remains controversial, as the expression analysis of membrane proteins in placental and CSA-binding isolates showed that neither *var*2CSA nor FCR2*var*CSA were preferentially expressed on the surface of IE (Fried et al., 2004).

Finally, the CD31/PECAM binding was reported to be mediated by CIDR1α, DBL2δ and the DBL5δ domain.

The phenotype associations and both host receptors and *P. falciparum* ligands involved are summarized in table 1.1.

Tabel 1.1. Host cell receptors and *P. falciparum* ligands involved in cytoadherence.

Host receptors	Receptor location	Parasite ligands (domain)
Sequestration		
TSP	Endothelium, serum	PfEMP-1
CD36	Endothelium, Erythrocyte	PfEMP-1 (CIDR1α)
ICAM-1 (CD54)	Endothelium	PfEMP-1 (DBLβ-C2)
PECAM-1 (CD31)	Endothelium, Monocyte	PfEMP-1 (CIDR1α and DBL2δ)
VCAM-1 (CD106)	Endothelium	?
ELAM-1 (E-selectin = CD62E)	Endothelium	?
P-selectin (CD62P)	Endothelium, Platelet	PfEMP-1
CSA	Endothelium	PfEMP-1 (DBL3γ, CIDR1α)
Rosetting		
A and B blood group	Erythrocyte	PfEMP-1 (DBL1α)
CR1 (CD35)	Erythrocyte	PfEMP-1 (DBL1α)
CD36	Erythrocyte, Endothelium	PfEMP-1 (CIDR1α)
HS	Endothelial, aorta	PfEMP-1 (DBL1α)
GAG	Erythrocyte	PfEMP-1 (DBL1α)
IgM and IgG	Serum	PfEMP-1 (CIDR1α, DBL2β)
Clumping		
CD36	Platelet	?
gC1qR/HABP1/p32	Platelet, Endothelium	?
P-selectin	Platelet	?

2. OBJECTIVES

1. Analysis of *P. falciparum var* gene sequences from Indonesian's field isolates

   Specific objectives of this topic:

   - To assess the overall diversity of *P. falciparum var* genes from Indonesian's field isolates.
   - To identify an association of restricted motifs in the DBL1α domain with clinical outcome in malaria.
   - To investigate a potential association of transcribed specific genotypes of *var* genes with the manifestation of severe malaria.

2. Analysis of *P. falciparum* DBLβ-C2 domains

   Specific objectives of this topic:

   - To explore the *P. falciparum* DBLβ-C2 sequences from Indonesian's field isolates.
   - To construct recombinant pDBLβC2 domains from severe and uncomplicated malaria patients and assess the binding capacity of recombinant pDBLβC2 with ICAM-1.

3. Detection of human *P. knowlesi* infection

   Specific objectives of this topic:

   - To identify naturally acquired human infections of *P. knowlesi* from Indonesian samples.
   - To investigate whether the available specific primers are sensitive and specific to diagnose *P. knowlesi* infection.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Study Site

The study outlined in this thesis was carried out at the Saiful Anwar hospital, Malang, East Java province and Pelaihari district, South Kalimantan province, Indonesia (Figure 3.1). The Saiful Anwar hospital serves as a referral hospital for the Eastern part of Indonesia, an area of moderate to high malaria endemicity. Most of the inpatient malaria cases in the Saiful Anwar hospital are imported cases from other areas such as Papua, Kalimantan or Nusa Tenggara, and the majority of patients suffered from severe malaria. Pelaihari district is located in South Kalimantan province, the region presents a range of different endemicity and environments, including coastal and hilly area with swamps and tropical climate. The climate is characterised by high temperature ranging between 15.6°C and 26.9°C and heavy rainfall ranging between 75 mm and 426 mm annually, especially during October to May. Humidity ranges between 77% and 91%.

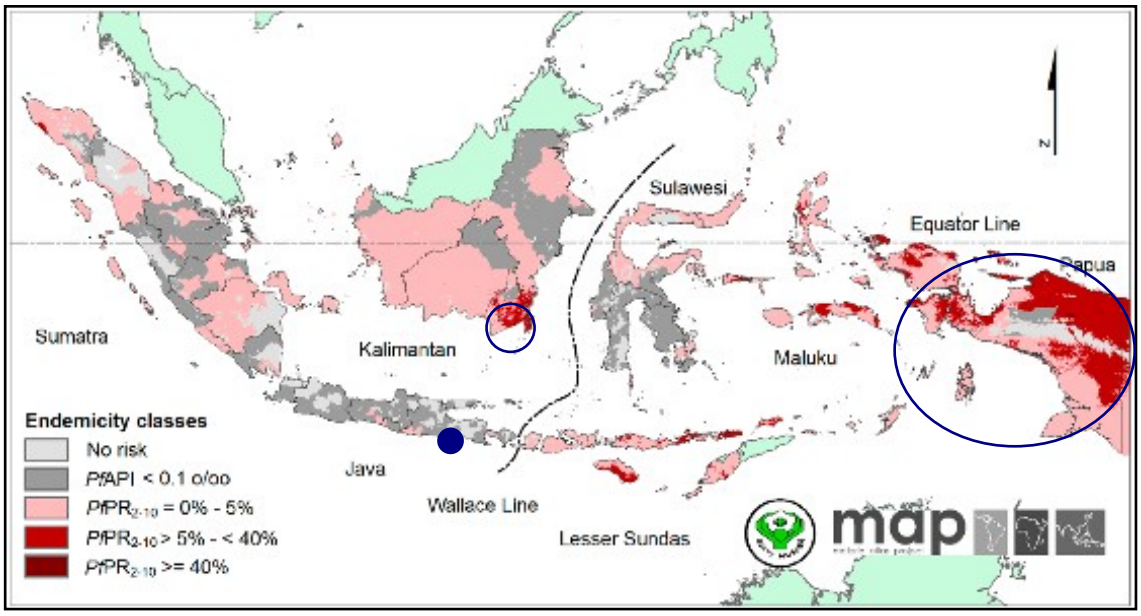


Figure 3.1. *P. falciparum* malaria endemicity class in Indonesia (Elyazar et al., 2011). The blue dot shows the hospital where severe malaria patients were recruited. The circled areas are originated location of the samples: South Kalimantan and Papua province, the areas of moderate to high malaria endemicity in Indonesia.

3.1.2 Sample collection

Blood samples were collected from *P. falciparum* malaria patients. Patients were categorised as having either severe or uncomplicated malaria based on the clinical and laboratorical criteria of WHO for malaria disease.

Inclusion criteria :

1. Patients age > 3 years old
2. Patients body weight >13 kg
3. Uncomplicated *P. falciparum* malaria: symptomatic malaria without signs of severity and/or evidence (clinical or laboratory) of vital organ dysfunction as found in severe malaria.
    - Parasite density <5%
    - Axillary temperature ≥ 37.5°C or recorded history of fever within the preceding 24 hrs
    - Symptoms: headache, lassitude, fatigue, abdominal discomfort, and muscle and joint aches, followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise.
4. Severe malaria: infection with *P. falciparum* with one or more of the following clinical symptom or laboratory test:
    - Coma (cerebral malaria) with abnormal behaviour, impairment of consciousness, seizures, coma, or other neurologic abnormalities
    - Prostration
    - Acute pulmonary oedema or acute respiratory distress syndrome (ARDS)
    - Circulatory collapse and shock, systolic blood pressure <70 mmHg in adult and <50 mmHg in children
    - Hepatic dysfuntion, clinical jaundice, bilirubin >3 mg%
    - Haemoglobinuria
    - Disseminated intravascular coagulation
    - Metabolic acidosis
    - Severe renal failure, creatinin >3mg%
    - Severe anaemia, haematocrit < 15% or Hb < 6g/dl
    - Hypoglycaemia, blood glucose < 40 mg/dl
    - Hyperparasitaemia ≥ 5%
5. Signed informed consent by the patient or the legal representative

Exclusion criteria:

1. Adequate anti-malarial treatment within previous 7 days
2. Mixed plasmodial infection

The study approved by Health Research Ethics Committee of the University of Brawijaya, Indonesia. Malaria patients were enrolled only after informed consent was

signed.

3.1.3 Reagents and Kits

Reagent	Company
Acetic acid	Merck, Darmstadt, Germany
Ampicillin sodium-salt	Sigma-Aldrich, Steinheim, Germany
β-mercaptoethanol	Sigma, Steinheim, Germany
Big dye terminatorTM mix (V3.1)	PE Applied Biosystem, CA, USA
Bovine serum albumin (BSA)	Sigma, Steinheim, Germany
Chelex 100	Biorad, Munich, Germany
d2H2O	Millipore, Eschborn, Germany
Deoxyribonuclease	Invitrogen, Carslbad, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
Dithiothreitol (DTT)	Invitrogen, Carlsbad, CA, USA
DMEM	Gibco, Paisley, UK
DNA ladder 1kb	Invitrogen, Carlsbad, CA, USA
DNA ladder 100 bp	Invitrogen, Carlsbad, CA, USA
DNase I	Qiahen, Hilden, Germany
dNTP mix (PCR grade)	GeneAmp, Applied Biosystem, USA
D-PBS	Gibco, Paisley, UK
Dye-Ex Spin kit	Qiagen, Hilden, Germany
Dynabeads M-280	Invitrogen, CA, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Steinheim, Germany
Ethanol 70%	Merck, Darmstadt, Germany
Ethanol 96%	Merck, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Fetal bovine serum (FBS)	Gibco, Paisley, UK
Ficoll (type 400)	Sigma, Steinheim, Germany
Flow fix, 2% paraformaldehyde	Polysciences, Eppelheim, Germany
Fluoromount-G	Southerbiotech, Birmingham, USA
GelRed Nucleic acid stain 10.000x in water	Biotium, Darmstadt, Germany
Gentamycinsulphat	Sigma-Aldrich, Steinheim, Germany
Giemsa stain	Sigma, Steinheim, Germany
Glucose	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
HEPES (N-(2- Hydroxyethyl) piperazine-N´- 2-ethanesulfonic acid)	Sigma, Steinheim, Germany
HiDi Formamide	Applied Biosystem, Warrington, UK
Hydrochloric acid (0.1 N)	Merck, Darmstadt, Germany
Hydrogen peroxide (H2O2) solution 35%	Sigma, Steinheim, Germany
Hypoxantine	Sigma, Steinheim, Germany
Isopropylthio-β-galactoside (IPTG)	Invitrogen, CA, USA
Isopropanol	Carl Roth, Karlsruhe, Germany
LB agar	Sigma-Aldrich, Steinheim, Germany
LB broth	Sigma-Aldrich, Steinheim, Germany
Lichrosolv water	Merck, Darmstadt, Germany
Lipofectin transfection reagent	Invitrogen, CA, USA
LongAmp Taq DNA polymerase	NewEngland Biolabs, Schwalbach, Germany
MgCl2	Sigma-Aldrich, Steinheim, Germany
Methanol	Merck, Darmstadt, Germany
OligodT primer	Invitrogen, CA, USA
Opti-MEM reduced serum medium	Gibco, Paisley, UK
pGEM-T Easy vector	Promega, Madison, USA
Phenol/chloroform/isoamylalcohol (25:24:1)	Merck, Darmstadt, Germany

Plasmid mini kit	Qiagen, Hilden, Germany
Polyethylenglycol (PEG) 4000	Merck, Darmstadt, Germany
Potassium acetate	Sigma, Steinheim, Germany
Qiamp DNA blood mini kit	Qiagen, Hilden, Germany
Qiamp RNA blood mini kit	Qiagen, Hilden, Germany
Qiashredder column kit	Qiagen, Hilden, Germany
QuickLyse miniprep kit	Qiagen, Hilden, Germany
Random hexamer primer p(dN)6	Invitrogen, CA, USA
Restriction enzymes	MBI Fermentas, St Leon-Rot, Germany
Restriction enzymes	NewEngland Biolabs, Schwalbach, Germany
RNeasy mini kit	Qiagen, Hilden, Germany
RPMI 1640	Sigma-Aldrich, Steinheim, Germany
SOC medium	NewEngland Biolabs, Schwalbach, Germany
SeaKem agarose	Cambrex Bio Science Rockland, USA
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Superscript II reverse transcriptase	Invitrogen, CA, USA
T4 DNA ligase	Invitrogen, CA, USA
Taq DNA polymerase	MP Biomedical, Germany
Tris (2-hydroxymethyl-aminomethan)	GE health care, Biosciences AB, Uppsala, Sweden
Triton-X	Carl Roth, Karlsruhe, Germany
Tryple express, stable trypsin	Gibco, Paisley, UK
Trypan blue	Sigma Aldrich, Steinheim, Germany
Tween 20	Merck, Hohenbrunn, Germany
Ultrafree-DA extraction kit	Millipore, Karlsruhe, Germany
Whatman filter paper	GE Health care, England
X-gal (5-Bromo-4-Chloro-3-indolyl a-Dgalactopyranoside)	Invitrogen, CA, USA

3.1.4 Sundry Materials

Material	Company
Cell Culture well	Corning, USA
Coverslip	EMS, USA
Cryotube vials	Nalge Nunc, Thermo scientific, Denmark
Centrifuge tubes	Corning, Mexico
Filter units	Millipore, Belford, USA
Inoculation loop, sterile	Greiner bio-one, Frickenhausen, Germany
Microcentrifuge tubes	Eppendorf, Hamburg, Germany
Microscope slide	Menzel-Gläser, Thermo scientific, Braunschweig, Germany
Needle	B. Braun, Emmenbruecke, Germany
Petridishes	Nunc, Thermo scientific, Germany
Pipette tips	Kisker, Steinfurt, Germany
Syringe	B. Braun, Emmenbruecke, Germany
Strypette	Corning, USA
Surgical scalpels	Aesculap, B.Braun, Tuttlingen, Germany
Tissue culture flasks	TPP, Switzerland

3.1.5 Buffers and Solutions

Buffers and solutions used in molecular biology

Name	Components
5x DNA Ligase reaction buffer	250 mM Tris-HCl pH 7.6

(Supplied with T4 DNA Ligase)	50 mM	$MgCl_2$
	5 mM	ATP
	5 mM	DTT
	25 %	PEG 8000
		Storage : -20°C
1x Tango buffer (Restriction enzyme buffer), Fermentas	33 mM	Tris-acetate pH 7.9
	10 mM	Mg acetate
	66 mM	Potassium acetate
	0.1 mg/ml	BSA
		Storage: -20°C
1x NE Buffer 4	50 mM	Potassium acetate
	20 mM	Tris-acetate
	10 mM	Mg acetate
	1 mM	DTT
		pH 7.9 at 25°C
		Storage: -20°C
1x LongAmp Taq reaction buffer	60 mM	Tris-SO_4
	20 mM	$(NH_4)_2SO_4$
	2 mM	$MgSO_4$
	3%	Glycerol
	0.06%	NP-40
	0.05%	Tween-20
		Storage: -20°C
1x PCR buffer with Mg	10 mM	Tris-HCl
	50 mM	KCl
	1.5 mM	$MgCl_2$
	0.1%	Triton X100
	0.2mg/ml	BSA
		Storage: -20°C
Buffer for plasmid preparation		
Solution I (resuspend buffer)	50 mM	Glucose
	25 mM	Tris-HCl pH 8.0
	10 mM	EDTA pH 8.0
		Storage : 2-8 °C
Solution II (lysis buffer)	1 %	SDS
	0.2 N	NaOH
		Storage : 15-25 °C
Solution III (neutralization buffer)	10 M	Potassium acetate pH 4.8
	5 M	Acetic acid
		Storage : 15-25 °C
10x TAE (Tris-acetate-EDTA) buffer	0.4 M	Tris
	0.2 M	Acetic acid
	10 mM	EDTA
		pH 8.3
		Storage : 20°C
		10x TAE buffer was purchased from Carl Roth, Karlsruhe, Germany
10x TAE buffer light	0.4 M	Tris-acetate
	1 mM	EDTA
		pH 8.3
		Storage : 20°C

		10x TAE buffer light was purchased from Carl Roth, Karlsruhe, Germany
10x BlueJuice Gel Loading buffer	65 % 10 mM 10 mM 0.3 % 	Sucrose Tris-HCl pH 7.5 EDTA Bromophenol blue Storage : 4 °C
TE (Tris-EDTA) buffer	10 mM 1 mM 	Tris-HCl EDTA pH 7.5, Autoclave Storage : RT

Buffers and solutions used in cell culture and immunofluorescence assay

Name		Components
Freezing medium	10% 90%	DMSO DMEM + 10% FBS
Binding medium	0.5 % 25 mM 	BSA HEPES In RPMI 1640 pH 7.0

3.1.6 Equipments

Instrument	Company
Centrifuge EBA 12	Hettich zentrifugen, Germany
Centrifuge Rotina 46	Hettich zentrifugen, Germany
Centrifuge mikro 22R	Hettich zentrifugen, Germany
Centrifuge Biofuge Pico	Heraeus, Germany
Fluorescence microscope Axioskop	Carl Zeiss, Goettingen, Germany
DNA analyzer ABI 3730	Applied Biosystems, CA, USA
Fridge 4°C	Liebherr, Germany
Freezer -20°C	Liebherr, Germany
Freezer -80 °C	Sanyo
Horizontal gel electrophoresis system	Peqlab Biotechnologie, Erlangen, Germany
Incubator	Heraeus, Germany
Inverted microscope	Leitz Labovert, Germany
Laminar flow Aura 2000 MAC	Bio Air Instruments S.r.l, Pavia, Italy
Liquid nitrogen tank	Air liquide Kryotechnik, Dusseldorf, Germany
Mini cenrifuge MCF-2360	LMS, Japan
Neubauer counting chamber	Marienfeld Superior, Lauda-Koenigshofen, Germany
pH meter	Sartorius, Germany
Phase-contrast microscope	Zeiss, Germany
Qubit fluorometer	Invitrogen, USA
Thermocycler T professional	Biometra, Goettingen, Germany
Thermomixer HLC MHL 20	HLC, Mannhein , Germany
UV transilluminator	Bachofer, Reutlingen, Germany
UV transilluminator	UVP, Cambridge, England
Vortex IKA MS3 basic	IKA, USA
Waterbath	Memmert, Germany
Waterbath	Julabo, Germany

3.1.7 Softwares

Name	Company	Usage
Adobe Photoshop CS2	Adobe System Inc.	Image processing

BioEdit v7.1.3	Tom Hall, Ibis Biosciences, Abbott laboratory, CA, USA	Analysis DNA sequences
DNASis Max v3.0	MiraBio Group, Hitachi Solution America, Ltd.	Analysis of DNA, RNA and amino acid sequences
EndNote X4	Thompson ISI, CA, USA	Bibliography management
Expasy translate tool	Expasy Bioinformatics Resource, Swiss Institute of Bioinformatics	Translation of DNA sequences into amino acid sequences
Grab-IT	Ultra Violet Product (UVP) Ltd, Cambridge, UK	Annotating image capture system
MS Office	Microsoft Corporation, USA	Text editing, data analysis and graph generation
NCBI-BLAST	National Library of Medicine, Bethesda, MD	Running and interpreting BLAST sequence comparison
Sequence scanner v1.0	Applied Biosystem, USA	View, edit, print and export data generated using the Genetic Analyzer
VarDom 1.0 server	Center for Biological Sequence analysis (CBS), Denmark	Composition and classification analysis of the malaria antigen family PfEMP-1

3.1.8 Culture medium

3.1.8.1 Parasite culture media

Incomplete medium:

10.43 g RPMI 1640 powder

2.00 g Glucose

0.05 g Gentamycin sulfate

5.94 g HEPES

0.04 g Hypoxanthine

Add distilled water to 1 liter, and adjust pH to 7.2. Filter-sterilized and store at -20°C.

Complete medium:

97.2 ml Incomplete medium

2.8 ml NaHCO3 (7.5%)

11-22 mL of human sera (inactivated at 56°C for 60 min, then stored at -20°C). The amount of serum depends on the age of the culture, in the first week medium should contain 20% serum, with the increasing stabilily of the culture, sera can be reduced to 10%.

5% erthrocyte suspension:

5 ml Packed cells

95 ml Complete medium

3.1.8.2 Bacterial culture media

LB medium:

1 % Tryptone

0.5 % yeast extract

0.5 % NaCl

1.5 % agar (for plates only)

pH 7.0

A ready to use mixture of the above components for LB-broth or LB-agar was used from Sigma. An appropriate amount of the powder was dissolved in d2H2O and autoclaved. In order to select transformants, ampicillin was added to the medium at final concentration of 50 μg/ml. The LB-agar medium with antibiotic was poured into Petridish and stored at 4°C.

3.1.8.3 Cell culture media

Complete medium: Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS.

3.1.9 Bacterial strains

Bacterial strain	Genotype	Company
E. coli DH5α	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end* A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ^-	Invitrogen, USA
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^qZΔM15]	Promega, USA

3.1.10 Mammalian cell line

COS-7 cells are immortalised fibroblast-like kidney cells from African green monkeys. This cell line is appropriate for transfection by vectors requiring expression of SV40 T antigen. It contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40°C and supports the replication of pure populations of mutants with deletions in the early region. The line was derived from the CV-1 cells (ATCC CCL 70) by transformation with an origin-defective mutant of SV40 which codes for wild type T antigen.

ATCC Number: CRL-1651

ECACC Number: 87021302

3.1.11 Plasmids

Name	Company	Usage
pGEM-T Easy	Promega, Madison, USA	Cloning vector with a single 3′-T overhangs at the cloning site for direct cloning of PCR products. It has α-peptide coding region of the enzyme β-galactosidase within the cloning region to allow identification of recombinant by blue/white screening.
pUC19	Invitrogen, USA	Cloning vector, used as transformation control.
pRE4	Prof. Gary Cohen and Prof. Roselyn Eisenberg, University of Pennsylvania, USA	Expression vector contains a SV40 *ori*, a Rous sarcoma virus LTR (RSV LTR) and the SV40polyA. It has the HSV glycoprotein D (HSV *g*D) used as the signal sequence and hydrophobic transmembrane to target different regions of the malarial erythrocyte binding proteins to the surface of mammalian cells.

3.1.12 Primers

Species-specific primers for *Plasmodium* spp.

1.	rPLU5	5′-CCT GTT GTT GCC TTA AAC TTC-3′
2.	rPLU6	5′-TTA AAA TTG TTG CAG TTA AAA CG-3′
3.	rFAL1	5′-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3′
4.	rFAL2	5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′
5.	rVIV1	5′-CGC TTC TAG CTT AAT CCA CAT AAC TGA TA-3′

6.	rVIV2	5′-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3′
7.	rMAL1	5′-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3′
8.	rMAL2	5′-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3′
9.	rOVA1	5′-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3′
10.	rOVA2	5′-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3′
11.	Pmk8	5′-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3′
12.	Pmkr9	5′-ACT CAA AGT AAC AAA ATC TTC CGT A-3′

P. falciparum genotyping primers

13.	M1-OF	5′-CTA GAA GCT TTA GAA GAT GCA GTA TTG-3′
14.	M1-OR	5′-CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA-3′
15.	M1-KF	5′-AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC-3′
16.	M1-KR	5′-GCT TGC ATC AGC TGG AGG GCT TGG ACC AGA-3′
17.	M1-MF	5′-AAA TGA AGG AAC AAG TCG AAC AGC TGT TAC-3′
18.	M1-MR	5′-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC-3′
19.	M1-RF	5′-TAA AGG ATG GAC CAA ATA CTC AAG TTG TTG-3′
20.	M1-RR	5′-CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC-3′
21.	M2-OF	5′-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′
22.	M2-OR	5′-CTT TGT TAC CAT CGG TAC ATT CTT-3′
23.	M2-ICF	5′-AGA AGT ATG GCA GAA AGT AAK CCT YCT ACT-3′
24.	M2-ICR	5′-GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG-3′
25.	M2-FCF	5′-AAT ACT AAG AGT GTA GGT GCA RAT GCT CCA-3′
26.	M2-FCR	5′-TTT TAT TTG GTGBCAT TGC CAG AAC TTG AAC-3′
27.	G-OF	5′-TGA ATT TGA AGA TGT TCA CAC TGA AC-3′
28.	G-OR	5′-GTG GAA TTG CTT TTT CTT CAA CAC TAA-3′
29.	G-NF	5′-TGT TCA CAC TGA ACA ATT AGA TTT AGA TCA-3′

Universal *var* primer to amplify the DBL1α

30.	αAF	5′-GCA CG(A/C) AGT TTT GC-3′
31.	αBR	5′-GCC CAT TC(G/C) TCG AAC CA-3′

Specific primer for *var* D-like gene

32.	*var*-DF	5′-AAT TCC T(C/G)A TGA (A/T)TT TAA (G/A)CG-3′
33.	*var*-DR	5′-CAC ATA ACA T(T/C)C C(A/T)T TCC A-3′

Primer pair to amplify conserved DBL domain

34.	UNIEBP-5	5′-CC(A/G) AG(A/G) AG(A/G) CAA (G/A)AA (C/T)TA TG-3′
35.	UNIEBP-3	5′-CCA (A/T)C(T/G) (T/G)A(A/G) (A/G)AA TTG (A/T)GG-3′

Sequencing primers for pGEMT-Easy vector

36.	SP6	5'-ATT TAG GTG ACA CTA TAG AAT AC-3'
37.	T7	5′-TAA TAC GAC TCA CTA TAG GGC GA-3′

Primer pair to amplify the whole DBLβ-C2 domain

38.	DBL2F1	5′-AGT GTG TTG AAG GAC GTA TGT-3′
39.	DBL2R3	5′-CCA AAC ATA TAT CTC TAT AAT CTC C-3′

Specific primer walking to sequence DBLβ-C2 domain

40.	DBLSeq_F	5′-ATG ACT GAA TGG GC(C/A) GAA TG-3′
41.	DBLSeq_R1	5′-CAA GAA GTC ATA CAC GGA T-3′
42.	DBLSeq_R2	5′-TAG TAC CAC CGA TTG AGC GT-3′
43.	DBLSeq_R4	5′-TAC ATT CTG GAT CCT CTT C-3′
44.	DBLSeq_R5	5′-TGT TCA TCG TCT TCA CCT T-3′
45.	DBLSeq_R11a	5′-CGTTGACTTGTGTACCACCA-3′
46.	DBLSeq_R11b	5′-ATG CGT CCT TAT ACT CTG G-3′

Specific primer to amplify the ICAM-1 binding portion of DBLβ-C2 domain

47.	DBL2_ICAM_F	5′-TCT CGT *CAG CTG* AGT GTG TTG AAG GAC GTA-3′
48.	DBL2_ICAM_R	5′-ACG AGT *GGG CCC* C(C/A)A AAG TTC TTT AAC TAT-3′
49.	DBL2_ICAM_R1	5′-ACG AGT *GGG CCC* ATT TGT GAC TAT AGT GCA-3′
50.	DBL2_ICAM_R4	5′-ACG AGT *GGG CCC* TCC TTC CAC AGT TTT ACA-3′
51.	DBL2_ICAM_R5	5′-ACG AGT *GGG CCC* AAG CAT TTT GTC CAC CGT-3′
52.	DBL2_ICAM_R11	5′-ACG AGT *GGG CCC* AAC TAT TTT GCA GGC AGG-3′

3.1.13 Antibodies

Antibody	**Company**	**Working dilution**
Dynabeads M-280 Sheep anti-	Invitrogen, USA	1-2x10^7

mouse IgG		beads/ml
DL6 monoclonal antibodies (mouse)	Prof. Gary Cohen and Prof. Roselyn Eisenberg, University of Pennsylvania, USA	1:2000
Fluorescein-conjugated goat anti-mouse antibodies	Jackson ImmunoResearch, USA	1:100
ID3 monoclonal antibodies (mouse)	Prof. Gary Cohen and Prof Roselyn Eisenberg, University of Pennsylvania, USA	1:2000
Mouse anti-human IgG Fc gamma fragment antisera	Jackson ImmunoResearch, USA	0.1-1µg Abs/beads
Recombinant human ICAM-1/CD54 Fc chimera	R and D System, Wiesbaden, Germany	1.5-2x10^6 beads

3.2 Methods

3.2.1 Cultivation of fresh *Plasmodium falciparum* isolates from patients

A sample of parasitized erythrocytes was collected from severe and asymptomatic or uncomplicated malaria patients. Blood was collected into tube containing heparin and saved at 4^{o}C until processed. Blood were layered by Ficoll and separated by centrifugation at 2000 rpm for 5 min. Erythrocytes were collected and washed three times in RPMI 1640. The isolates were grown in complete medium and supplemented with 5% erythrocyte suspension. Cultures were maintained at 37^{o}C in an atmosphere of 94% nitrogen, 5% carbon dioxide and 1% oxygen, and the culture medium is changed every day. The growth rate was monitored by microscopic examination of Giemsa-stained smears.

Sorbitol-synchronization of *P. falciparum* culture

The parasite culture was harvested at the ring stage (10-12 h post invasion culture). The culture was centrifuged at 1200 rpm for 2 min, the pellet were added with the same volume of 5% sorbitol and incubated for 10 min at RT, with 2 or 3 times shaking. The culture was centrifuged, washed 3 times in complete medium and diluted to 5% erythrocyte suspension. The culture was counted and subcultured.

3.2.2 Microbiology techniques

3.2.2.1 Bacterial cultures

The frozen cells from the glycerol stocks or resuspension of lyophilized cells were streaked out on to LB-agar plates containing appropriate selective antibiotics and incubated o/n (over-night) at 37°C to obtain single colonies. A single colony was then inoculated in the LB medium containing appropriate antibiotic and incubated o/n at 37°C in a shaking incubator at 250 rpm. To prepare competent bacterial cells, LB agar and medium without antibiotics were used.

3.2.2.2 Preparation of bacterial stocks

850 µl of an overnight bacterial culture was mixed with 150 µl sterilized glycerol and store at -80°C for long term storage.

3.2.2.3 Preparation of chemically competent bacteria

A single bacterial colony was inoculated in 50-100 ml of LB medium and incubated at 37°C with shaking at 250 rpm. The culture was grown until the cell density reached an OD_{600} of 0.4-0.5, it took for approximately 3-6 hours. In order to stop the bacterial growth, the cultures were put on ice immediately. The cells were harvested by centrifugation at 2500 xg for 15 min at 4°C and resuspended in 1/10 volume (5-10 ml) of ice cold TSS and place on ice. The competent cells were aliquoted (50-100 µl vol.) and stored at -80°C.

For some cloning experiments, highly efficient chemically competent *E. coli* DH5α cells and *E. coli* JM109 cells were purchased from invitrogen and promega, respectively.

3.2.3 Molecular biology techniques

3.2.3.1 DNA extraction

DNA extraction from patients whole blood and *P. falciparum* culture

Genomic DNA was isolated from whole blood of malaria patients and parasite culture by QIAamp DNA Blood mini kit (Qiagen) according the manufacture's instruction. 200 µl of whole blood or of harvested uniformly ring-stage parasite culture by centrifugation at 300 xg for 5 min was diluted with 200 µl PBS, then added with 20 µl proteinase-K. The mixture were added with 200 µl buffer AL, vortexed for 15 s and incubated at 56°C for 10 min. The samples were precipitated with 200 µl ethanol 96-100% and applied to the QIAamp Mini spin column to centrifuge at 8000 rpm for 1 min. Then added with 500 µl buffer AW1 and centrifuged at 8000 rpm for 1 min. The procedure was continued by

adding the sample with 500 µl buffer AW2 and centrifuged at full speed for 3 min. Finally, the samples were dissolved by adding 200 µl buffer AE and centrifuging at 8000 rpm for 1 min. DNA was stored at -20°C.

DNA extraction from blood on filter paper

DNA extraction from blood on filter paper was conducted using Chelex boiling method. Chelex was gently mixed by magnetic stirer for 10 min before used. The blood spot on filter paper was cut for approximately 1 cm^2 and put into 1.5 ml tube. The sample was dissolved with 200 µl prepared Chelex and mixed well by vortexing for 30 sec. The sample was cooked by heating at 100°C for 15 min and centrifuged at 12.000 rpm for 2-5 min. The supernatant was collected and centrifuged at 12.000 rpm for 2-5 min. 80-100 µl of supernatant was collected and stored at -20°C.

3.2.3.2 RNA extraction

RNA extraction from patients whole blood and *P. falciparum* culture

Total RNA was isolated from whole blood and parasite culture using QIAamp RNA blood mini kit according to the manufacturer's protocol. 500 µl of whole blood or harvested synchronized parasite culture by centrifugation at 300 xg for 5 min was mixed with 5 volume of buffer EL and incubated on ice for 10-15 min. The sample was centrifuged at 400 xg at 4°C for 10 min and the supernatant was discarded. The sample was lysed again by adding 2 volume of buffer EL and centrifuged, and added 350 µl of buffer RLT to the pellet before applied to the QIAshredder spin column and centrifuged at maximum speed for 2 min. The sample was added with 1 volume of 70% ethanol and applied to the QIAamp spin column and centrifuged for 15 sec at >10000 rpm. The sample was washed with 700 µl buffer RW1 and added with 500 µl buffer RPE and centrifuged for 15 sec >10000 rpm, and once again with 500 µl of buffer RPE, centrifuged at maximum speed for 3 min. Finally RNA was diluted with 30-50 µl of RNase-free water and centrifuged for 1 min at >10000 rpm. RNA was used immediately or stored at -80°C.

RNA extraction from blood on filter paper

RNA was also extracted from dried blood on filter paper using RNeasy Mini Kit (Qiagen). The blood spot was cut for approximately 0.3 cm^2 and placed into the tube. 600 µl of RLT buffer was added and incubated in thermomixer at 1000 rpm 37°C for 30 min.

The lysate was applied to the QIA shredder spin column and centrifuged at 13.000 rpm for 2 min. 1 volume of 70% ethanol was added to the homogenized lysate and mixed properly by pipetting. 700 µl of lysate was applied to the RNeasy spin column and centrifuged for 15 sec at ≥10.000 rpm. The sample was washed by adding 350 µl of buffer RW1 to the column and centrifuged for 15 sec at ≥10000 rpm. 10 µl DNase I stock solution was mixed with 70 µl buffer RDD and the mix solution was applied to the spin column membrane and incubated at RT for 15 min. The column was washed again with 350 µl buffer RW1 and centrifuged at ≥10000 rpm for 15 sec. The sample was added with 500 µl buffer RPE, centrifuged for 2 min at ≥10000 rpm and centrifuged once again at maximum speed for 1 min. Finally diluted RNA by adding 30-50 µl of RNase-free water directly to spin column membrane and centrifuging for 1 min at ≥10000 rpm. RNA was used immediately or stored at -80°C.

3.2.3.3 cDNA synthesis

A complementary DNA (cDNA) is a DNA copy synthesized from RNA. cDNA synthesis is used to determine the level of gene expression. cDNA is synthesized first using oligodT or random primers in the present of four deoxynucleotide triphosphates (dNTP) by using reverse transcriptase with RNA or mRNA as a template reaction, followed by PCR.

RNA was treated with DNase I for 15 min at 37°C to avoid contamination with genomic DNA. The mixture contained of 1ng-5µg total RNA, 1 µl of 500µg/ml OligodT primer and 1 µl dNTP mix (10mM each) was heated to 65°C for 5 min and quick chilled on ice. 4µl of 5x first-strand buffer and 2 µl of 0.1 M DTT was added to the mixture and incubated at 42°C for 2 min. The mixture was then reverse transcribed using 1 µl (200 U) of Superscript II RT by incubating at 42°C for 50 min and the reaction were inactivated by heating at 70°C for 15 min. The cDNA was used as a template for subsequent PCR and stored at -20°C.

3.2.3.4 Polymerase Chain Reaction (PCR)

The PCR is a technique for amplifying a piece of DNA of interest in an exponential manner. The amplification requires synthetic oligonucleotides, so called primers, which flanking the target sequence to be amplified. These primers, under appropriate conditions, anneal to the denatured target sequence and, in the presence of a heat stable DNA polymerase enzyme and dNTPs, direct synthesis a complementary strand of the

target sequence. As a template 1 µl cDNA (10-200 ng) or 1 µl genomic DNA (0.1-1 µg) or 0.5 ng plasmid DNA were used. The PCR reactions were prepared as follows:

PCR component	Amount (µl) 20 µl per reaction	Final concentration
10x PCR buffer	2.0	1x
25 mM MgCl2	1.2 – 2.0	1.5 – 2.5 mM
10 mM dNTP mix	0.4	200 µM
10 µM forward primer	0.4 – 1.0	0.2 – 0.5 µM
10 µM reverse primer	0.4 – 1.0	0.2 – 0.5 µM
DNA template (0.5 ng/µl to 1 µg/µl)	1 - 5	N.A
DNA polymerase (5U/µl)	0.2 – 0.3	1 – 1.5 U
ddH2O	Adjust to 20	

Some reactions were also performed in 50 µl. And the cycle conditions were as follows:

Step	Temperature (°C)	Duration (min:sec)	No. of cycles
Initial denaturation	94-95	4:00	1
Denaturation	94-95	0:45 - 0:60	25 - 35
Annealing	42-65	0:60	
Extension	65-72	1:00 - 3:00	
Final extension	65-72	10:00	1

The annealing temperatures are varied between 42 and 65°C depend on the length and Tm (melting temperature) of the primer pairs, it should be 5°C below the lowest Tm of the pair of primers. The extension temperatures depend on the DNA polymerase used, and the duration of extension varied according to the length of the target sequence with the rule of 1 min per 1000 bp fragment.

***Plasmodium* species-specific identification by PCR**

Species-specific identification was conducted with nested PCR. All PCR amplifications were carried out in a 20 µl reaction mixture:

PCR component	Amount (µl)	Final concentration
10x PCR buffer + MgCl2 (1.5 mM)	2.0	1x
100 mM dNTP mix	0.1	500 µM
10 µM forward + reverse primer	0.5	0.25 µM/primer
DNA template (0.5ng/µl to 1 µg/µl)	1.0	N.A
DNA polymerase (5U/µl) (MP Biomedical)	0.08	0.4 U
ddH2O	Adjust to 20	

The amplification was performed in a thermocycler (Biometra, Germany) as follows:

Step	Temperature (°C)	Duration (min:sec)	No. of cycles
Initial denaturation	95	5:00	1
Denaturation	94	1:00	25
Annealing	58	2:00	
Extension	72	2:00	
Final extension	72	5:00	1

DNA of *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi* were included as positive controls. One microliter of the nested 1 amplification product served as DNA template of each of the 20 µl nested 2 amplification. Another components of the reaction mixture were identical with nested 1. Nested 2 amplification conditions were identical to those of nested 1 except for *P. knowlesi* which used the annealing temperature of 60°C and 30 cycles.

3.2.3.5 DNA analysis and purification

Determination of DNA/RNA concentration

DNA concentration was measured using Qubit Fluorometer, fluorescence-based dyes that bind specifically to DNA, RNA and protein. The purity of the sample was determined by the OD260/280 ratio. The pure DNA sample should have a ratio between 1.8 and 2.0. Smaller ratios usually indicate contamination by protein or organic chemicals.

Agarose gel electrophoresis

Agarose gel electrophoresis was applied for DNA separation. It is used for both analysis and isolation of the DNA fragments. The concentration of agarose gel was determined according to the desired range of separation of DNA fragment. Higher concentrations facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNA fragment, as shown in the table below:

% agarose	Range of separation for linear DNA
0.5	1 – 30 kb
0.7	0.8 – 12 kb
1.0	0.5 – 10 kb
1.2	0.4 – 7 kb
1.5	0.2 – 3 kb
2.0	0.05 – 2 kb

The gel was prepared by boiling the desired concentration of agarose in 1x TAE buffer. After cooled to approximately 50^{o}C, 1 µl of GelRed Nucleic acid stain was added per 10 ml of gel volume. The gel was poured onto a horizontal gel-tray and waited until completely cooled before transferred into appropriate gel chamber. 8-10 µl DNA samples or DNA marker were loaded in the gel slots with loading dye added to 1x final concentration. The DNA fragments were separated by applying an electrical field 60-100 V, under these condition the negatively charged DNA molecules would migrate to the anode at rates that are inversely proportional to the length of the individual DNA fragments. Larger molecules migrate slower than smaller molecules. The gels would typically be run for 1-2 hours. The detection was done using UV light transilluminator (302 nm), and the gels were documented using Grab-IT program.

DNA purification from agarose gel

DNA fragments that would be used in the cloning experiments or directly sequencing were cut out from the gels and extracted from the gel using Ultrafree_DA extraction kit (Millipore) according to the manufacturer's instruction. The excised gel slice was transferred to the column and centrifuged at 5000 rcf for 10 min. The DNA was stored at -20^{o}C.

3.2.3.6 DNA digestion using restriction enzymes

Restriction endonuclease cuts the DNA at a short specific nucleotide recognition site. For site-specific digestion of DNA, type II restriction endonucleases are required. The enzymes are able to recognize, bind and cleave the phosphodiester bond at short specific nucleotide sequence within DNA target. The digestion of DNA was performed with specific buffers that had a various compositions of salts and buffering agents depending on the restriction enzyme used. When double digest were applied, an appropriate buffer for both enzymes were used according to the manufacturer's instruction. For single digestion the following components were mixed and the digestion was allowed for 1 hour at enzyme-dependent temperature:

Reaction component	Amount
Appropriate enzyme Buffer	1x final concentration
Restriction enzyme	5 - 10 units
DNA	0.5 - 1µg
Nuclease free-water	To a final volume of 50 µl

3.2.3.7 Ligation

Ligation reactions were performed to ligate the DNA fragments from vector and insert (PCR products). The ligation reactions of PCR products with pGEMT-Easy vector were performed according to the manual. The vector and insert were used at a molar ratio of 1:1 to 1:3 (vector : insert) in the ligation reaction as described in the following table. The reaction was incubated o/n at 4°C or 1-2 hours at RT.

Reaction component	Amount (µl)
2x Rapid Ligation buffer	5
pGEMT-Easy vector (3 kb in size; 50 ng)	1
Insert DNA (0.5 kb in size)	x
T4 DNA Ligase (3 U/µl)	1
Nuclease-free water	Adjust to 10

3.2.3.8 Transformation of plasmid DNA into bacterial cells

The transformation was performed according to the manufacturer's instuctions. Briefly, the competent cells were thawed on ice for 5-10 min. 5 µl control DNA (50 pg) was

pipetted in a pre-chilled tube containing 100 µl competent cells and 2-3 µl ligation reaction in 50 µl competent cells, mixed by gentle flicking. The mixtures were incubated on ice for 30 minutes, heat-shocked in 42°C water bath for 45 sec and immediately chilled on ice for 2 min. The transformed cells were resuspended in pre-warmed SOC medium to a volume of 1 ml. The cultures were incubated at 37°C with 250 rpm shaking for 60-90 min, then 100-200 µl transformation reaction was plated on LB agar containing ampicillin, IPTG and X-gal. The plates were incubated o/n at 37°C.

3.2.3.9 Plasmid DNA isolation

Plasmid DNA was isolated from bacteria using the alkaline lysis method (Sambrook, 1989). Some plasmid mini preparation kits (Qiagen plasmid mini kit) were also purchased. The plasmid isolation was performed according to the manufacturer's instructions. A single white *E. coli* colony was inoculated into 3 ml of LB medium containing selective antibiotic and incubated at 37°C with vigorous shaking for approximately 8 hours. The starter cultures were diluted 1/500 to 1/1000 into 3 ml selective LB medium and incubated at 37°C with vigorous shaking for 12-16 hours. The cultures were harvested by centrifugation for 15 min at 8000 rpm and the supernatant was discarded. The pellet was resuspended in 300 µl of P1 buffer and the cells were lysed by adding 300 µl of P2 buffer and incubated at RT for 5 min. The lysate was neutralized with 300 µl of P3 buffer and incubated on ice for 5 min. The reaction was centrifuged at maximum speed for 10 min and the supernatant containing plasmid DNA was applied to a Qiagen-tip. The Qiagen-tip was washed with the buffer QC and DNA was eluted with the buffer QF. The DNA was precipitated with 0.7 volume of RT isopropanol, mixed and centrifuged at >10.000 rpm for 30 min. The DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 10.000 rpm for 10 min. The pellet DNA was air-dried, dissolved in 50µl of TE buffer and stored at -20^{o}C.

3.2.3.10 Sequencing

DNA sequencing was applied to confirm the identity of a clone and obtain the exact sequence. For all sequencing, the Sanger chain termination method was used. The sequencing was performed with a cycle-sequencing dye-dideoxy chain termination protocol. The cycle sequencing reaction consists of successive rounds of denaturation, annealing and extension in the presence of primer, DNA polymerase, dNTPs and dye-labeled terminator dideoxy nucleotides (ddNTPs). For the cycle sequencing reaction, the

following components were added:

Reaction component	Amount (µl)
Sequencing buffer	1.5
BigDye terminator v3.1	0.5
Primer (10 µM)	1
PCR product or plasmid DNA	10-100 ng PCR product or 0.5-1.0 µg plasmid DNA

The reaction was performed in a thermocycler with the following program:

Step	Temperature (°C)	Duration (min:sec)	No. of cycles
Denaturation	96	00:30	25
Annealing	50	00:15	
Extension	60	04:00	

After completed cycle sequencing, 15 µl ddH2O was added to reach a volume of 20 µl. This mixture was cleaned-up using a DyeEx 2.0 spin column (DyeEx 2.0 spin kit, Qiagen) and centrifuged at 2700 rpm for 3 min according to the manufacturer's instructions. This step efficiently removes the excess dye terminators from the completed DNA sequencing reactions. The purified sequencing reaction was then precipitated with 3 volume of ethanol 96% and twice with 2 volume of ethanol 70%. All precipitation reactions were carried out by centrifugation at maximum speed (in g or rpm) for 3 min. After drying for approximately 45 min, the samples were diluted with 12 µl HiDi Formamide plus 13 µl Lichrosolv water and loaded in a 96-well sample plate of a DNA Analyzer (ABI 3730, Applied Biosystems) for sequence analysis.

3.2.3.11 Sequence analysis

The nucleotide sequences were aligned using BioEdit program and DNASIS MAX version 3.0 and analyzed for sequence similarities by NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were translated into amino acid sequences using Expasy Translation Tool (http://www.expasy.ch/tools/dna.html). The multiple sequences were aligned using ClustalW alignment. Percentage sequence similarity test and phylogenetic tree analysis

were carried out using the algorithm in DNASIS MAX version 3.0 based upon a ClustalW alignment.

3.2.4 Cell culture techniques

3.2.4.1 Cultivation of mammalian cells

The mammalian cell line (COS-7 cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and incubated in a 37^{o}C incubator containing a humidified atmosfer of 5% CO_2. For harvesting the adherent cells, the 80-100% confluent cultures were washed with D-PBS and treated with pre-warmed Trypsin-EDTA for 5 min to detach the cells from the surface of culture flask and separate the cells from each other. The activity of the trypsin was stopped by adding complete medium (DMEM+10%FBS) and the cells suspension was either used for subculturing or for the preparation of frozen stocks. The cell lines were propagated and maintained by seeding at the density of 1-2 x 10^4 viable cells/cm^2 every 3-5 days depending on the growth characteristic of the cells.

3.2.4.2 Freezing and thawing of mammalian cells

Mammalian cell lines can be maintained by storing them in liquid nitrogen. For freezing, 80-100% confluent culture were pelleted by centrifugation at 100 x g for 5 min and resuspended in chilled freezing medium containing complete medium and 10% volume of DMSO to reach approximately 5 x 10^6 viable cells/ml. The cells suspension were transferred into cryo tubes and then put in a -80°C freezer to cool the cells about -1°C/min. After 24 hrs, they can be stored in liquid nitrogen.

The frozen cells can be revived by proper thawing. For optimal recovery, rapid thawing was important, therefore the cells were thawed rapidly in a 37°C waterbath and washed with 10-20 ml D-PBS or culture medium and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in fresh pre-warmed DMEM supplemented with 10% FBS. The cell lines were cultured for a minimum of 3 passages before used in other experiments.

3.2.4.3 Determination of cell viability

To determinate cell viability, we used trypan blue exclusion assay. Trypan blue stain distinguishes viable cells from non-viable cells by their capability to absorb the dye, viable cells actively exclude the trypan blue while non-viable cells absorb dye. For counting the number of viable cells, the cell suspension was diluted with trypan blue at a ratio of 1:1 to 1:9 (cell suspension:trypan blue) and the number of viable cells in a 4 x 1

mm^2 area (4 x 100 nl? µl? of diluted cell suspension) were counted with the help of a Neubauer haemocytometer under a phase contrast microscope. The total number of viable cell per ml of the cell suspension was calculated using the following formula:

$$\text{Total number of viable cells /ml} = \left(\frac{\text{number of viable cells in 4x 1mm}^2\text{ area}}{4}\right) \text{x dilution factor x } 10^4$$

3.2.4.4 Transient transfection of adherent cells

Transient transfections of adherent cells were performed by using a transfection reagent (Lipofectin, Invitrogen). One day before transfection, COS-7 cells were plated into coverslip in six-well plates with 2 ml DMEM supplemented with 10% FBS. The cells were incubated for 16-24 hours in a humidified 5%-CO_2 atmosphere at 37°C until 60-80% of the well bottom was covered by cells. 3-5 µg of plasmid DNA was diluted with 100 µl OPTI-MEM reduced serum and mixed with the dilution of 10 µl lipofectin in 100 µl OPTI-MEM reduced serum, then incubated for 15 min at RT. Meanwhile, the cells were washed once with 2 ml DMEM without serum. The DNA-complex were added with 800 µl OPTI-MEM without serum and added to the cells and incubated at 37°C in a CO_2 incubator for 5-24 hours. The medium was replaced with 2 ml complete growth medium (DMEM+10% FBS). The transfected cells were assayed for expression and binding activity by immunofluorescence assay after 24-72 hours post transfection.

3.2.5 Immunofluorescence techniques

3.2.5.1 Immunofluorescence assay to test transfection efficiency and expression of construct on the surface of COS-7 cells

The transfected COS-7 cells were washed twice with PBS and fixed in 2% paraformaldehyde for 15 min in RT then washed again in PBS. The fixative cells were stained with the primary antibody (ID3 or DL6) at a dilution of 1:2000 in PBS containing 0.1% BSA for 1 hour at RT. The cells were washed with PBS for 10 min and incubated with fluorescein-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratory) at a dilution of 1:100 in PBS containing 0.1% BSA for 1 hour. The cells were washed again with PBS for 10 min, mounted with Fluoromount (Southern Biotechnology Associates Inc) and air dried for 5 minutes before examination. The surface expression was scored using a fluorescence microscope. The mAbs (ID3 or DL6) were tested against untransfected COS-7 cells to check for the background fluorescence.

3.2.5.2 Immunofluorescence assay to assess the ICAM-1 binding of the recombinant construct pDBLβ-C2

Coating Dynabead M-280 sheep anti-mouse IgG with ICAM-1

Dynabeads M-280 sheep anti-mouse Ig G antibody (Invitrogen) were washed before use to remove preservatives, facilitated by the use of a magnet as recommended by manufacturer. The Dynabeads M-280 were resuspended thoroughly in the vial by vortexing and transfered the required amount of resuspended beads into a new tube. The tube was placed on the magnet for 2 min and pipetted off the supernatant. After removing the tube from the magnet, the pellet was resuspended in an excess volume of washing buffer (0.1% bovine serum albumin in PBS). The tube was placed again on the magnet, pipetted off the supernatant and resuspended the washed beads in any choosing volume of washing buffer with the final concentration of 1-2 x 10^7 beads/ml.
Washed Sheep anti-mouse Ig G antibody-coated magnetic Dynabeads were incubated with mouse anti-human IgG Fc gamma fragment antisera (Jackson ImmunoResearch) at a concentration of 0.1-1.0μg antibody/10^7 beads. Beads were mixed by rotation for 3 h at RT. The tube containing the mixture was placed on the magnet for 2 min and pipetted off the supernatant, than removed from the magnet and washed with washing buffer. The tube was placed again on the magnet, removed the supernatant and washed with washing buffer. After coating with mouse anti-human IgG Fc gamma fragment antisera, the beads were than coated with recombinant ICAM-1/Fc protein (R&D Systems) at ratio of 1.0μg ICAM-1/10^7 beads (per 1 x 10^6 cells), or mock treated for controls by overnight incubation. The beads were washed with washing buffer and resuspended in binding medium (0.5% bovine serum albumin, 25 mM HEPES in RPMI-1640 pH 7.0) at a concentration of 3.7 x 10^7 beads/ml and warmed to 37°C prior to adding the cells.

Assessing binding of recombinant construct pDBLβ-C2 with ICAM-1 coated beads

Binding of recombinant construct pDBLβ-C2 with ICAM-1 coated beads was tested at pH 7.0. Two days (40-60 h) post-transfection, transfected COS-7 cells in coverslips were transferred to an empty fresh well of six-well plate (Falcon) and overlaid with 40 μl of binding medium (0.5% BSA, 25mM HEPES in RPMI 1640) containing 1.5 – 2.0 x 10^6 beads of ICAM-1-coated or mock-coated. The cells were incubated in a humidified container for 1 h at 37°C and filled with 10 ml of binding medium at the same pH used during the incubation of beads and cells (pH 7.0). The coverslips were then flipped cell-side down onto a stand and incubated for 5 – 10 min to allow unbound beads to settle by

gravity. Coverslips were then returned to cell-side up and examined under phase microscopy to determine the degree of beads association.

To prove that beads specifically associated with transfected cells, coverslips were fixed in 2% paraformaldehyde (Polysciences) for 15 min at RT. The fixed coverslips were processed for immunofluorescence as previously described (2.2.5.1) to analyze the coincidence of recombinant protein expression and bead association.

Binding was expressed as a percentage of transfected cells containing five or more ICAM-1 coated beads. Experiments were carried out in duplicate with a minimum of 50 transfected cells were analyzed for each construct. Standard deviation was calculated using Microsoft excel (Springer et al., 2004).

3.2.6 Statistical analysis

Standard statistical analysis (eg. mean, median and standard deviation) was calculated using the built in statistic functions of Microsoft Excel program. The differences between sequence groups were analyzed using Fisher's Exact and Chi-square tests. All tests were two-tailed and results considered significant if $p<0.05$ meaning that there is a 95% chance that the two groups being compared would be truly different if looking at the entire population of parasites.

4. RESULTS

4.1 Characteristic of samples

Twelve Indonesian malaria patients with written informed consent were enrolled in this study. All patients were adults, five suffered from clinically severe malaria and seven were uncomplicated malaria. Severe patients were hospitalised in the Saiful Anwar hospital in Malang who came either from Papua or South Kalimantan province. Uncomplicated cases were recruited from Pelaihari district, South Kalimantan. All severe cases had an impairment of consciousness along with one or more severe clinical manifestations defined by WHO including severe anaemia, respiratory distress, renal failure, hepatic dysfunction and jaundice. One patient was pregnant in the third trimester, the case was considered as a pregnancy-associated malaria (PAM). Patients had symptoms up to 14 days before they were recruited. Patients were microscopically diagnosed as mono-infection of *P. falciparum*, severe malaria patients had parasitaemia ranging between 1% and 24.6%, parasitaemia of uncomplicated cases ranged between 0.2% and 2% (Table 4.1).

Table 4.1. Profile of malaria patients

Sex	Age (years)	Origin	Parasitaemia (%)	Disease state group
Severe malaria				
M	35	Papua	10	Cerebral malaria, anaemia, respiratory distress, renal failure
M	50	South Kalimantan	1	Cerebral malaria, clinical jaundice, anaemia
F	19	Papua	12.8	Cerebral malaria, clinical jaundice, severe anemia, PAM
M	47	South Kalimantan	2	Cerebral malaria, anaemia
M	51	Papua	24.6	Cerebral malaria, clinical jaundice, renal failure, hepatic dysfunction
Uncomplicated malaria				
M	35	South Kalimantan	1.1	Mild malaria
M	41	South Kalimantan	0.2	Mild malaria
M	19	South Kalimantan	1	Mild malaria
M	54	South Kalimantan	1	Mild malaria
M	16	South Kalimantan	1	Mild malaria
M	35	South Kalimantan	1	Mild malaria
M	55	South Kalimantan	2	Mild malaria

4.2 Identification of *Plasmodium* species

In order to molecularly confirm mono-infection of *P. falciparum*, *Plasmodium* species-specific PCR was conducted by using specific primers of five different *Plasmodium* spp causing human malaria (3.1.12). Eight out of twelve patients were detected as mono-infection with *P. falciparum*, three samples were mixed infection with *P. falciparum, P. vivax* and *P. knowlesi*, and one sample was mixed infection of *P. vivax* and *P. knowlesi.* Neither *P. malariae* nor *P. ovale* were detected in the samples. Due to the inclusion criteria, we excluded the samples with mixed-infections. Only eight samples of *P. falciparum* mono-infection were included in the further analysis.

4.3 Detection of human *Plasmodium knowlesi* case

Because of the origin of the samples, they were examined for *P. knowlesi*, the proposed fifth humanpathogenic Plasmodium. Amplification by using specific primer Pmk8 and Pmkr9 (3.1.12), designed for *P. knowlesi*-specific DNA sequences on the *small subunit ribosomal* RNA (ssrRNA) gene, yielded a single band of 153 bp in four samples (Figure 4.1). All *P. knowlesi*-positive samples were mixed infection with *P. vivax* and/or *P. falciparum*. None of them were positive in the *P. malariae* specific PCR. They had clinically mild malaria with a low range of parasitaemia between 0.2% and 1% and were microscopically diagnosed as mono-infection of *P. falciparum* (Table 4.2). *P. knowlesi*-positive samples came from South Kalimantan province, the Indonesian part of Borneo, none from Papua.

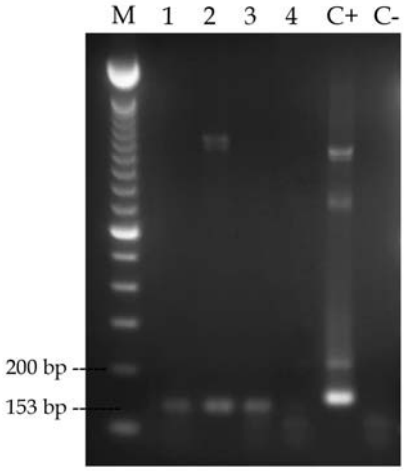


Figure 4.1. Amplification results using Pmk8 and Pmkr9 primers to detect *P. knowlesi* case. Four positive samples numbered as 1 to 4 generated a 153 bp band. M: marker 100 bp, C+: positive control, C-: negative control.

Table 4.2. Profile of *Plasmodium knowlesi*-positive patients

Sex	Age (years)	Clinically	Microscopy -base diagnosis	PCR-base diagnosis	Sequence analysis of *P. knowlesi* 153 bp band
M	35	Mild malaria	*P. falciparum*	*P. falciparum*, *P. vivax*, *P. knowlesi*	*P. vivax*
M	41	Mild malaria	*P. falciparum*	*P. falciparum*, *P. vivax*, *P. knowlesi*	*P. vivax*
M	54	Mild malaria	*P. falciparum*	*P. falciparum*, *P. vivax*, *P. knowlesi*	*P. vivax*
M	16	Mild malaria	*P. falciparum*	*P. vivax*, *P. knowlesi*	*P. knowlesi* (GU233448)

PCR using *P. knowlesi*-specific primers (Pmk8 and Pmkr9) yielded a single band of 153 bp and direct sequencing of the PCR products were performed. Sequencing showed a perfect matching with the recently published *P. knowlesi* S-type from Malaysian Borneo and Vietnam in one of the four samples (Figure 4.2). The other sequences were repeatedly consistent with the ssrRNA gene of the *P. vivax* S-type, blasting results presented higher homology (93-100%) with various *P. vivax* strains than with different *P. knowlesi* (maximum of 82% homology) or other Plasmodia strains

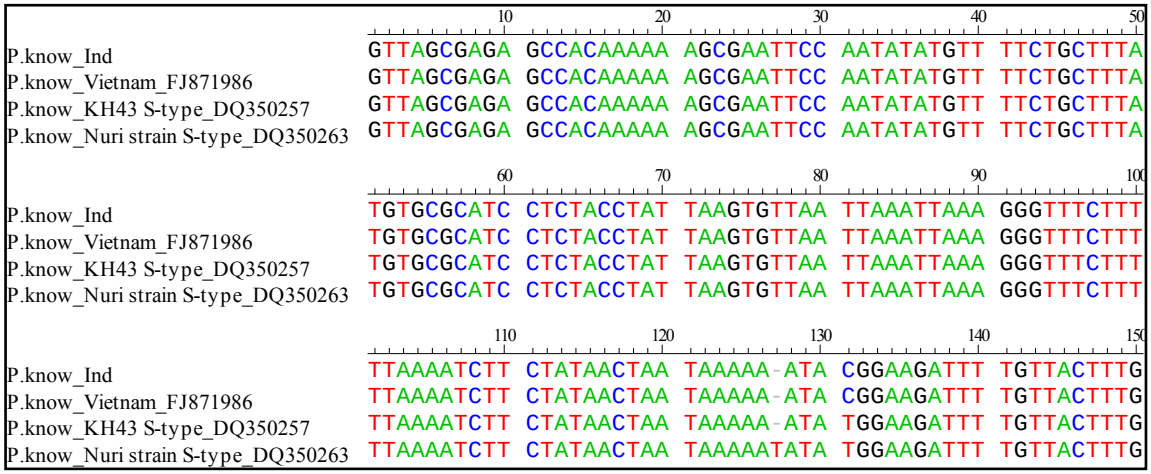


Figure 4.2. Multiple alignment of *P. knowlesi* ssRNA from Indonesia with other *P. knowlesi* strains. Indonesian *P. knowlesi* sequence showed a 99-100% homology with *P. knowlesi* S-type from Malaysia and Vietnam. Compared to *P. knowlesi* Nuri strain S-type, there was only one nucleotide 'T' deletion in 127 bp.

4.4 Genotyping of *Plasmodium falciparum*

P. falciparum-mono infection samples were genotyped using the highly polymorphic repetitive regions of MSP-1, MSP-2 and GLURP genes. Multiple fragments were detected from all allelic families (Table 4.3). Ten different bands in the MAD20 allelic family were found, 0-4 in each sample; the fragment size ranged from near 160 bp to approximately

350 bp. The K1 allelic family showed four bands between 180 bp and 350 bp, each sample presented maximum two. The RO33 allelic family showed two fragments of approximately 160 bp and 180 bp solely in two samples (Kal1 and Kal3). By using MSP-2 genotyping, six different bands of the 3D7/IC family were detected, 0-4 in each sample ranging from approximately 400 bp to 800 bp. The FC27 family presented ten different bands, ranging from 270 bp to 600 bp, 0-6 per sample. 1–2 bands in each sample were observed by using GLURP specific primers with seven different bands between 600 bp and 1.1 kb. Amplified bands of MSP-1, MSP-2 and GLURP presenting a similar fragment size were considered clonally identical.

The multiplicity of infection (MOI), which is defined as the minimum number of genetically different parasite lines (clones) present in each sample was calculated by counting the number of bands observed for each genetic markers of three distinct families of MSP-1 and two distinct families of MSP-2, and the highest number between two genetic markers is considered as the MOI of the sample. The MOI of samples were in a range of 2 and 10 parasite lines for each sample where the samples from severe cases tended to have a higher MOI (4-10 parasite lines) than samples from uncomplicated cases (2-3 parasite lines).

Table 4.3. Genotyping results by MSP-1, MSP-2, and GLURP

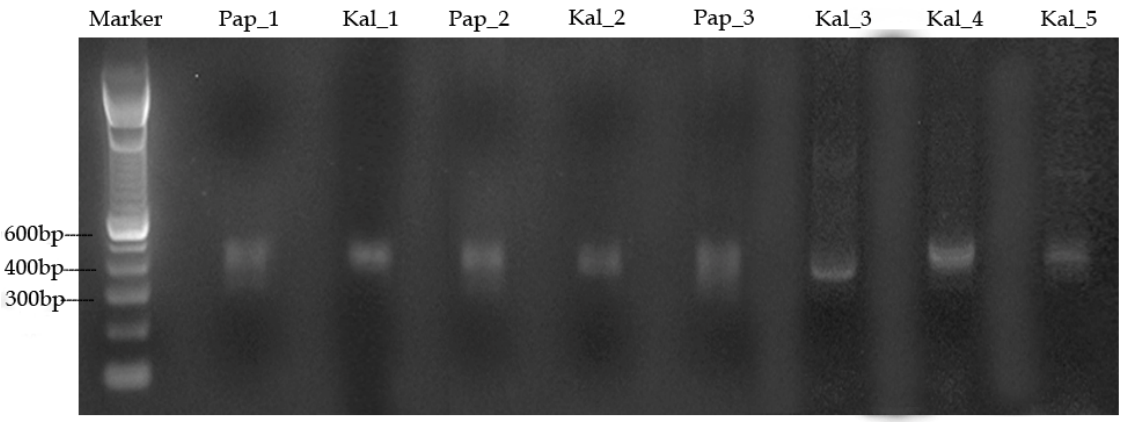
Sample	Malaria clinical	Number of MSP-1 bands			Number of MSP-2 bands		Number of GLURP bands	MOI *
		MAD 20	K1	RO33	3D7/IC	FC27		
Pap1	Severe	4	2	0	1	4	1	**6**
Kal1	Severe	1	0	1	2	2	2	**4**
Pap2	Severe	4	2	0	3	0	1	**6**
Kal2	Severe	3	2	0	1	6	1	**7**
Pap3	Severe	4	1	0	4	6	2	**10**
Kal3	Uncomplicated	0	1	1	1	1	1	**2**
Kal4	Uncomplicated	2	0	0	0	2	2	**2**
Kal5	Uncomplicated	1	1	0	1	2	1	**3**

*MOI = multiplicity of infection is defined as the minimum number of genetically different parasite lines (clones) present in each sample.
MOI was calculated by counting the number of variants (bands) observed for each of genetic markers, for MSP-1 the bands observed for the MAD20, K1 and RO33 families are added and for MSP-2 the bands detected for the 3D7/IC and FC27 are added. The highest number between two genetic markers is considered as the MOI of the sample.

4.5 Diversity of *var* genes

var genes diversity was determined by amplification of both genomic DNA and cDNA using universal primer αAF and αBR (3.1.12). The design of the primers was based on the high conservation in flanking regions of DBL1α, the amino-terminal DBL domain. Since the DBL1α is present in most family members of the *var* gene, the primer pair amplifies the majority of *var* gene repertoire in both laboratory and field isolates. Amplification resulted a single fragment of ~370 - 450 bp in each sample both from genomic DNA and cDNA (Figure 4.3). Amplified bands were then cloned into cloning vector pGEMT-Easy to allow identification of every single DBL1α sequence within amplified fragments. It was attempted to characterise the majority of DBL1α sequences from each sample. Sequencing was conducted twice for each clone from both strands using SP6 and T7 primers located within the vector.

(a)



(b)

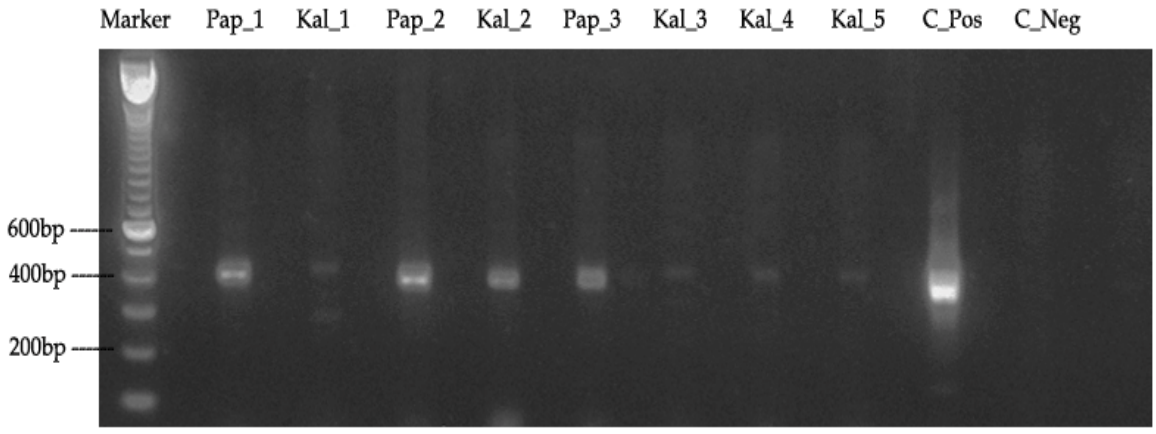


Figure 4.3. Amplification results of universal primer αAF and αBR. Each sample generated a single band of ~370–450 bp; (a) Amplification fragment from genomic DNA. (b) Amplified band of cDNA synthesised from RNA of blood on filter paper; C_Pos: positive control; C_Neg: negative control.

Derived from eight field isolates, where three originated from Papua and five from South Kalimantan province, seventy-one different sequences out of total 104 DBL1α sequenced clones were identified with less than 95% sequence similarity. Twelve different sequences derived from parasite cDNA of four severe and one of uncomplicated malaria patients. Others were amplified from parasite genomic DNA of all patients. On average, 8.9 different DBL1α sequences per isolate were discovered.

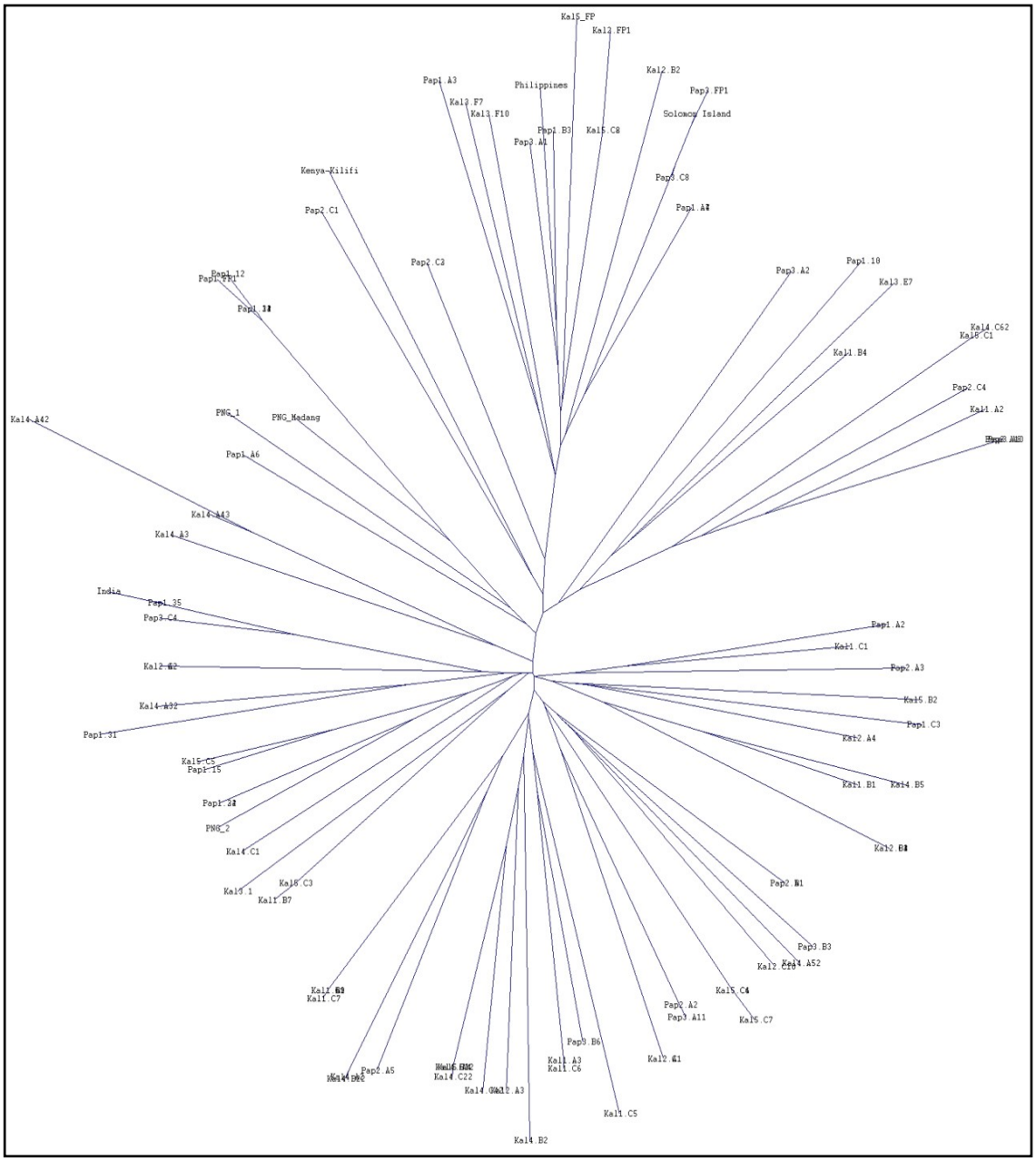


Figure 4.4. **Unrooted phylogram of DBL1α sequence tag from Indonesian field isolates and other global isolates.** Four pairs (Kal4.C62 - Kal5.C1; Kal4.B12 - Kal5.A4, Kal1.B7 - Kal5.C3; Pap2.A2 - Pap3.A11) of DBL1α sequences presented more than 95% similarity. Each pair was detected in two different isolates but originated from the same geographical area. Two sequences from Papua (Pap1.13 and Pap3.C8) showed more than 95% similarity with isolates from India and Solomon Islands.

The average DBL1α amino acid sequence similarity within isolates ranged from 47.2 ± 6.8 to 51.8 ± 5.9 and among isolates from 42.4 ± 6.5 to 49.4 ± 6.8. Four pairs of DBL1α sequences presenting more than 95% similarity were detected, where three pairs were found in the two isolates from Kalimantan (Kal4.C62 and Kal5.C1; Kal4.B12 and Kal5.A4; Kal1.B7 and Kal5.C3) and one similar sequence was observed in Pap2.A2 and Pap3.A11 from Papua isolates. In comparison with published sequences, only two sequences from Papua (Pap1.13 and Pap3.C8) showed more than 95% similarity with isolates from India and Solomon Islands, respectively. Phylogenetic analysis demonstrated no clustering of sequences regarding strain or geographical origin (Figure 4.4).

4.6 Determination of distribution motif in DBL1α

4.6.1 Classification of the DBL1α sequence tag

The DBL1α sequence tag regions start at a DIGDI motif within homology block (HB) 'd' and end at a PQFLR motif within HB 'h' of the Smith's classification (2000[b]). The sequence tags are classified into six sequence groups based on the number of cysteine residues within the tag region and a set of sequence motifs at four positions of limited variability (PoLV1-4) as proposed by Bull et al. (2007). The positions within the sequences are fixed in relation to four anchor points. PoLV1 and PoLV4 are defined in relation to the 5′ and the 3′ ends of the sequence, respectively. PoLV2 and PoLV3 are correlated to a "WW" motif. This classification is referred to as a cysteine/PoLV sequence grouping and described as follows:

- Group 1: cys2, PoLV1(MFK*)
- Group 2: cys2, PoLV2(*REY)
- Group 3: cys2 not groups 1, 2
- Group 4: cys4 not group 5
- Group 5: cys4, PoLV2(*REY)
- Group 6: cys1, 3, 5, >5

The DBL1α sequences were also characterised by recently described homology blocks (HB)s using the VarDom 1.0 server (http://www.cbs.dtu.dk/services/VarDom/), a server dedicated particularly to analyse composition and classification of the malaria antigen family PfEMP-1. Homology Block is defined as a member of sequences with similarity to the sequence profile above a certain threshold (9.97) as determined by Rask and colleagues (2010). This HB classifications are also redefined the Smith's HB a-j classification, where HBb=HB4, HBd=HB3, HBf=HB5, HBh=HB2 and HBj=HB1, but

based on the similarity threshold, the remaining Smith's HB are not found to be conserved in all DBL classes.

4.6.2 Sequence distribution motif of DBL1α

Analysis of DBL1α sequence showed a conservation of certain residues in DBL1α domain. The conserved regions were interspersed within variable blocks and vary in both length and sequence. A cysteine/PoLV sequence classification presented a similar distribution of all sequence groups between isolates causing severe and uncomplicated malaria (p=0.48), but all sequences of group 3 were obtained from severe cases. Comparison of the individual sequence groups demonstrated that there was strong evidence for a difference between sequence group 3 and group 4 (p=0.02) and between sequence group 3 and group 6 (p=0.03). Sequence group 4 which has 4 cysteine residues, termed as a normal number of cysteine within DBL1α sequence tag, was identified as the most frequent sequence in both severe and uncomplicated malaria patients (Figure 4.5).

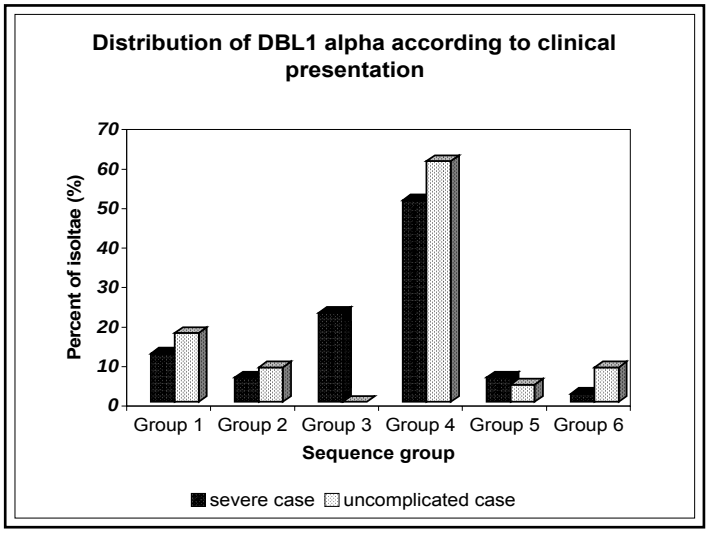


Figure 4.5. Distribution of DBL1α sequences into six sequence groups according to the clinical presentation. Parasite isolates from both severe and uncomplicated malaria patients showed a similar distribution of DBL1α sequence, with a notification that all sequences group 3 were obtained from severe cases. The majority of sequences from both clinical cases belong to sequence group 4.

In addition, analysis of DBL1α sequence tag in association with the geographical origin by using the cysteine/PoLV sequence grouping indicated no evidence for a specific difference between isolates from Papua and South Kalimantan province (p=0.18).

Similar to the report by Bull et al. (2007) concerning the association between PoLV motifs and distinct sequence length distributions, sequences containing the MFK* at PoLV1

(group 1) or the *REY at PoLV2 (group 2 and 5) were associated with short sequences (Figure 4.6).

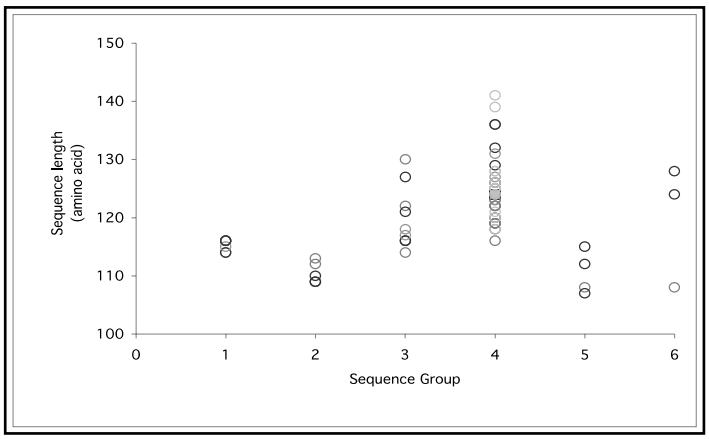


Figure 4.6. Association of PoLV motifs with distinct sequence length distributions. Two PoLV motifs were independently associated with short sequence i.e. MFK* at PoLV1 for sequence group 1 and *REY at PoLV2 for sequence group 2 and 5.

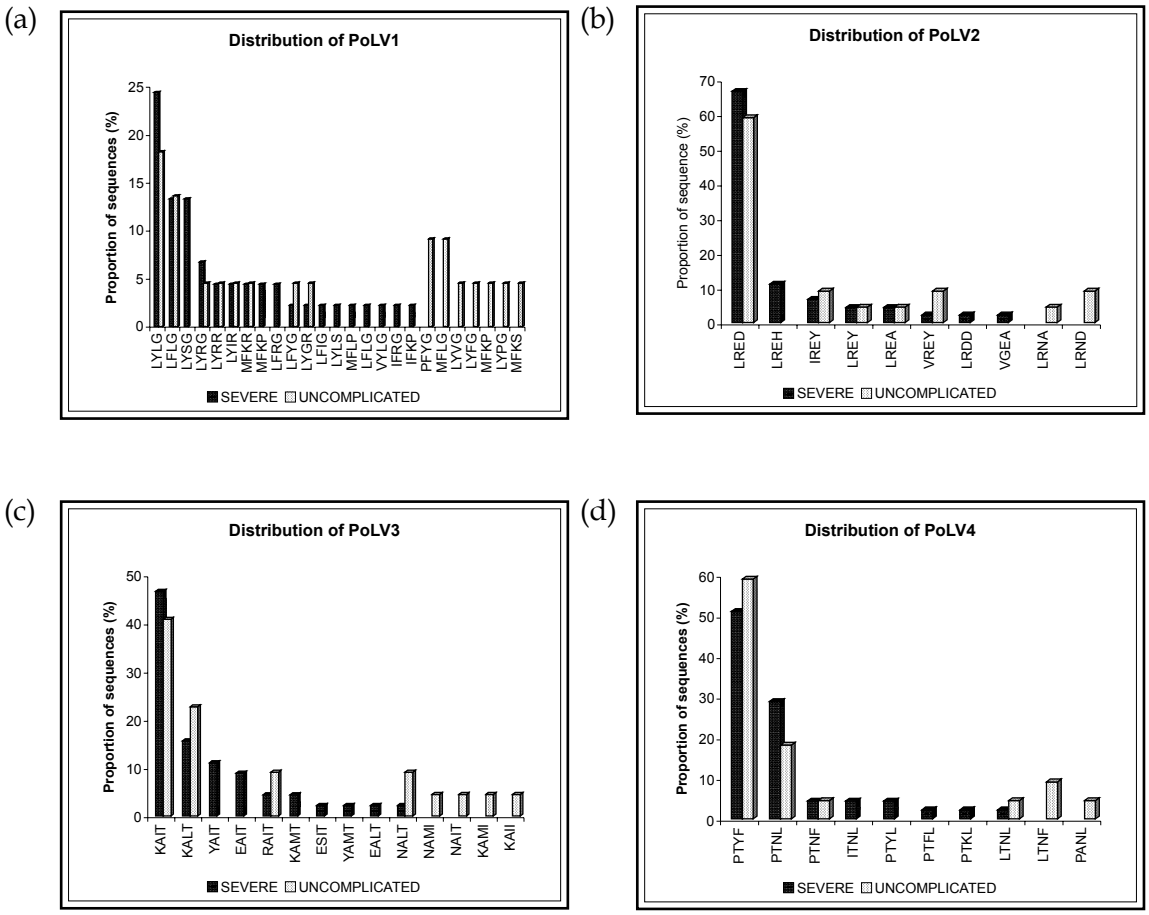


Figure 4.7. Distribution of PoLV1-4 motifs within isolates causing severe and uncomplicated malaria. Each PoLV has one motif as a major motif that was observed in both severe and uncomplicated cases. Some motifs were detected solely in severe or uncomplicated malaria. (a) The most frequent motif of PoLV1 was LYLG. (b) In PoLV2 , LRED was the major motif. (c) Most PoLV3 motifs were KAIT. (d) Main motif in PoLV4 was PTYF.

One motif in each PoLV was revealed as the major motif and observed in both severe and uncomplicated cases, i.e. LYLG in PoLV1, LRED in PoLV2, KAIT in PoLV3 and PTYF in PoLV4. Some motifs were detected solely in either severe or uncomplicated malaria (Figure 4.7).

Analysis using varDom server showed that our DBL1α sequences stretched from the most prevalent HB3 to HB2, including subdomain S2b and S2c. It showed a presence of HB36 in all sequences containing 4 cysteine residues (cys4) but absence in all sequences containing 2 cysteine residues (cys2) as described previously (Rask et al., 2010). HB60 was found in almost all cys2 sequences and in 11 of 51 (21.6%) cys4 sequences. The same percentage of cys4 sequences presented also HB88 that were absent in cys2 sequences. HBs 2, 3, 5, 14, 54, 60, 64, and 131 were found in both sequence types. HB36, HB79, and HB88 could only be detected in cys4 sequences (Appendix 1 and 2).

4.6.3 Distribution motif in expressed *var* DBL1α sequence

Nine different DBL1α sequences amplified from cultured parasite cDNA of one severe malaria case were identified. Four sequences showed similarities of 97-99%, differing minimally in HB5 and 14 and might be considered as the same. *var* gene expression was also investigated by RNA extraction of dried blood on filter paper. The sequences were mostly similar to those derived from the genomic DNA of each isolate.

Analysis using a cysteine/PoLV classification of cultured parasite cDNA (Pap1) showed sequence group 2 once, sequence group 3 five times and sequence group 4 three times (Table 4.4). Meanwhile, two *var* gene sequences from blood filter paper of severe cases belonged to sequence group 1 and two to sequence group 3. But, solely one case of uncomplicated malaria showed a DBL1α sequence tag by amplification belonging to sequence group 1. Amplification of two other uncomplicated cases yielded single bands but sequencing results showed less than 12% similarity with DBL1α.

Tabel 4.4. Distribution motif of expressed *var* DBL1α sequences

RNA Source	Cysteine/PoLV grouping (cys2)			Cysteine/PoLV grouping (cys4)		Cysteine/ PoLV grouping (cys others)
	Group 1 (sequence)	Group 2 (sequence)	Group 3 (sequence)	Group 4 (sequence)	Group 5 (sequence)	Group 6 (sequence)
Filter paper	**3**	**0**	**2**	**0**	**0**	**0**
Culture	**0**	1	**5**	**3**	**0**	**0**

All expressed sequences extracted from filter paper of severe cases and two third extracted from culture of severe patient consisted of only two cysteine residues as shown in table 4.4, consistent with presence of HB 60 and absence of HB 36 (Appendix 2). Three sequences derived from cDNA of the cultured parasites presented 4 cysteine residues (cys4) and showed HB 36 in three sequences and HB60 in two sequences (Appendix 1).

4.7 Identification of conserved DBL domains

DBL domains were also identified from genomic DNA and cDNA by using degenerate UNIEBP primers (3.1.12). The primers were designed for the relatively conserved motifs of DBL domains. Amplification of genomic DNA resulted in multiple fragments ranging from nearly 250 bp to 1 kb in all samples as presented in figure 4.8. Samples originating from the same geographical area tended to present similar patterns of amplified bands i.e. Pap1, Pap2 and Pap3 which came from Papua and Kal1, Kal2, Kal3, Kal4 and Kal5 which originated from South Kalimantan. Samples from South Kalimantan generated more amplified fragments then those of from Papua. Sequencing of the 525 bp band corresponded to a *var* gene coding for DBLγ isolated in Gabon, the 445 bp fragment matched with a *var* gene coding for PfEMP-1 from an isolate associated with placental malaria in Malawian women.

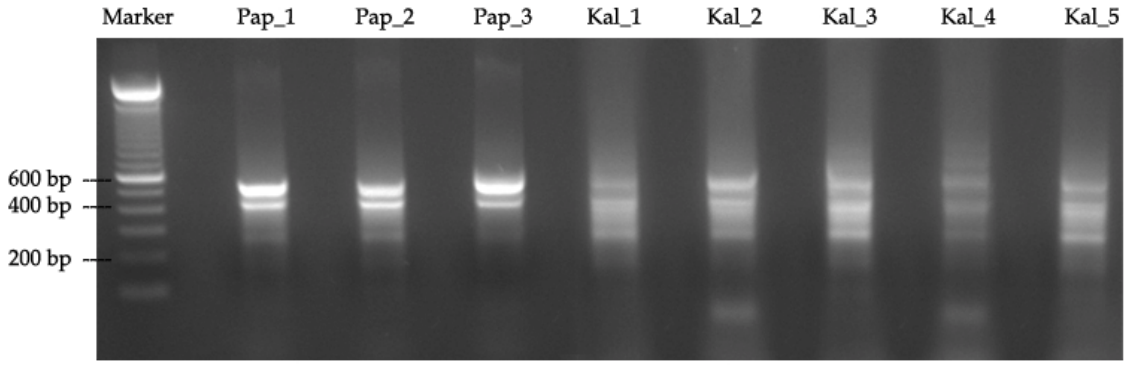


Figure 4.8. Amplification of genomic DNA by using UNIEBP primers. Each sample yielded multiple bands ranging from nearly 250 bp to 1 kb. Samples from the same geographical area tended to present similar pattern of amplified fragments.

Amplification of cDNA generated one to four bands ranging from 250 bp to approximately 600 bp in four out of five severe malaria patients, and no band observed from cDNA of uncomplicated cases although they had quantifiable RNA. Sequencing of a 418 bp fragment matched with the *Plasmodium falciparum eba-175* gene and a 316 bp corresponded to DBL1α domain. A 486 bp band matched with the DBLγ domain isolated

from placenta of Malawian women with PAM and var1CSA of 3D7. This band was only found in a male patient from Papua (Pap3).

4.8 Detection of *var* D-like gene

To investigate the association of expressed specific motifs with severe malaria outcome, *var* D-like specific primers (3.1.12) were designed based on the 525 bp fragment of the PCR product that resulted from UNIEBP primers, as reported by Ariey and colleagues (2001). Amplification using *var* D-like specific primers resulted in multiple fragments from genomic DNA of all samples, but generated a single ~237 bp fragment of expressed sequences from four severe malaria patients only. Sequence identity to the previously reported *var* D gene (AJ277137) (Ariey et al., 2001) was 29.0, 27.8, 27.0 and 19.3 % for *var* D-like sequence of Kal2, Pap3, Pap1 and Pap2, respectively.

Our *var* D-like sequences showed 80-94% identity with the DBLγ fragment of PfEMP-1 from several isolates including 3D7, Malawian isolates from patients with pregnancy-associated malaria (PAM), placental isolates from Gabon, Kenya and Malawi (Figure 4.9). However, further analysis showed three conserved motifs containing 6-10 nucleotides within all four of our sequences (Figure 4.9).

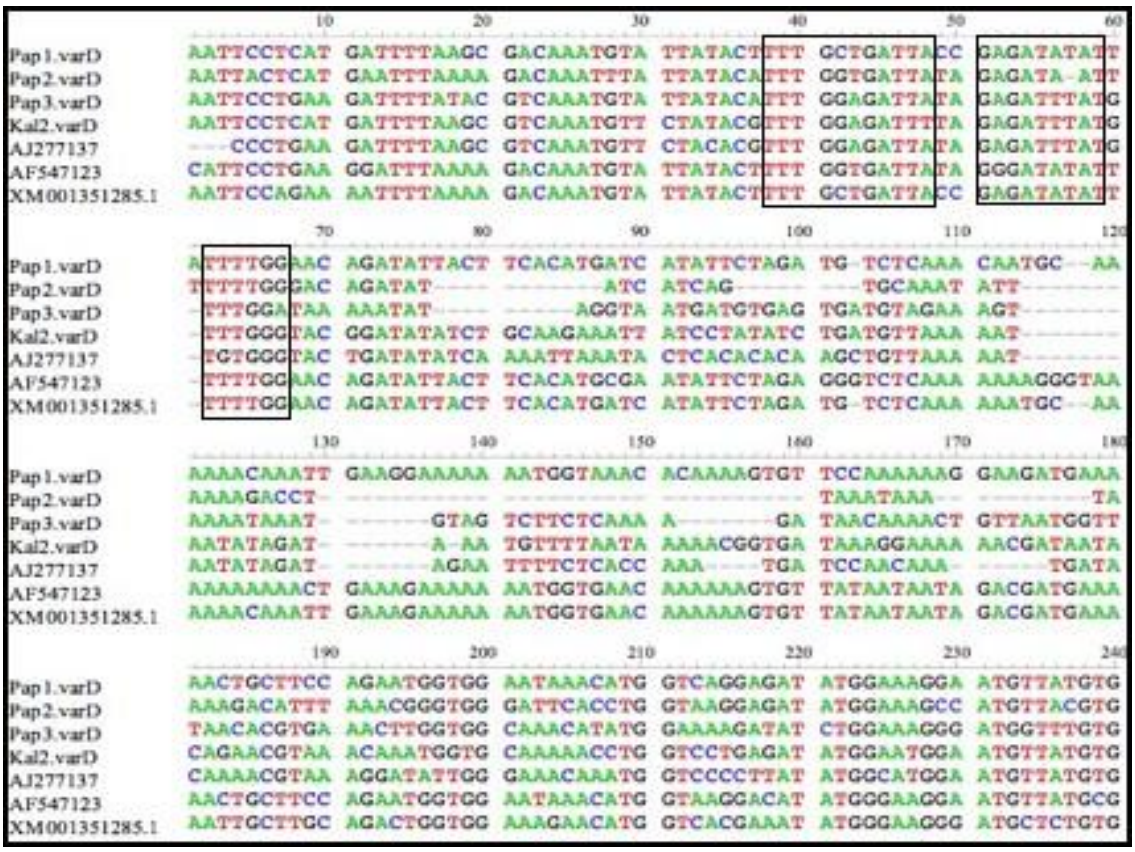


Figure 4.9. Multiple aligment of specific *var* D-like sequences. Specific primers designed for the Indonesian *var*-D like sequence resulted in sequences corresponding to different sequence types including the DBLγ domain of PfEMP-1 isolated from 3D7 [XM_001351285] and patients in association with PAM [AF547123]. Sequence identity of 29.0, 27.8, 27.0 and 19.3 % was shown for *var* D-like sequence of Kal2, Pap3, Pap1 and Pap2 respectively to the previously reported *var* D gene (AJ277137). Black boxes: conserved motifs containing of 6-10 nucleotides.

Analysis using varDom 1.0 server showed the conserved motif in HB3 and HB5, one sequence shared HB86 with the previously reported *var* D gene (Table 4.5). Of interest, HB86 was mainly found in DBLγ domain while the proposed *var* D gene (AJ277137) was reported to correspond with DBLδ domain. Another sequence showed HB27 and HB98, one other sequence presented HB82 and the rest showed only HB3.

Table 4.5. Distribution of homology blocks (HBs) among the proposed *var* D gene using VarDom server

Sequence	Score HB3	Score S2b subdomain					HB5
		HB82	HB86	HB98	HB27	HB624	
Pap1.*var* D-like	-	26.5	-	-	-	-	16.4
Pap2.*var* D-like	24.6	-	-	-	-	-	-
Pap3.*var* D-like	31.3	-	-	30.1	14.7	-	22.5
Kal2.*var* D-like	35.5	-	32.0	-	-	18.1	-
AJ277137 (*var* D)	37.4	-	32.6	-	-	-	32.2

4.9 Identification of DBLβ-C2 domain of PfEMP-1

Specific primer for the DBLβ-C2 sequence were designed according to several referred sequences of the DBLβ-C2 domain (2.1.12): JDP8 (AY028643), isolate IT4/24/25 (IT-ICAM var) (AY578326), clone A4 strain IT4/25/5 (L42244.1), clone A4tres isolate IT (AF193424) and FCR3var CSA (AJ133811). After a series of PCR optimisation, amplification of genomic DNA generated 1-2 fragments in most samples, except for isolate Pap3 that presented additional bands of 500 bp and 600 bp, but all samples had a band of nearly 1.7 kb. Two samples had a similar pattern of amplified fragments (Figure 4.10).

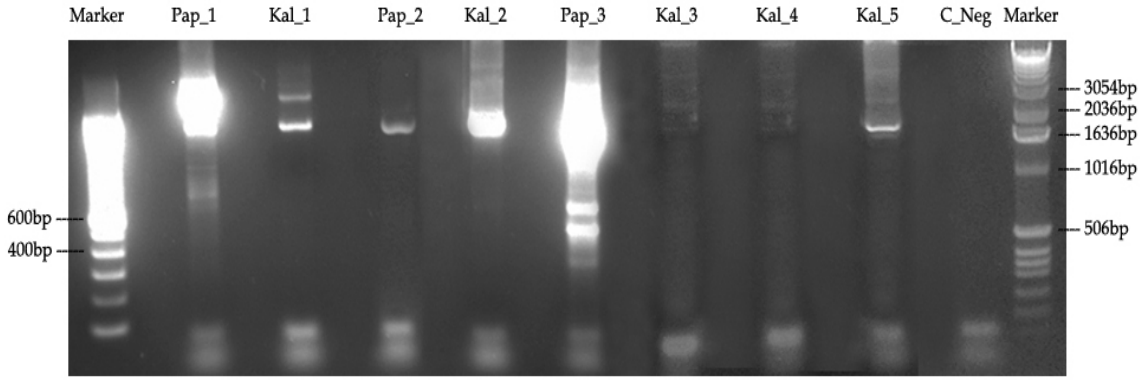


Figure 4.10. Amplification results using specific DBLβ-C2 primer from genomic DNA of field isolates. All samples had a band nearly 1.7 kb with one or two additional bands around 3 kb (Pap1, Kal1) or 600 bp and 500 bp (Pap3). Kal3 and Kal4 presented similar pattern of amplified band. A ~1.7 kb fragment was used for cloning to identify the DBLβ-C2 sequences of field isolates.

Considering the length of DBLβ-C2 domain from referred sequences, we took the ~1.7 kb band and cloned them into a pGEMT-Easy vector. Four to seven clones for each sample were sequenced twice from both strands using SP6 and T7 primers. Due to the length of DBLβ-C2 sequences, we did primer walking by designing specific primers of the DBLβ-C2 sequence of each sample in order to identify the inner part of the sequences (3.1.12).

Derived from eight field samples, ten different sequences out of thirty-seven sequenced clones were identified (Figure 4.10). Each sample possessed one DBLβ-C2 sequence type, except for Pap2 and Pap3 had two sequence types. Three DBLβ-C2 sequences from three different samples (Kal1, Kal3, and Kal5) presented more than 99% sequence similarity, all three samples were originated from the same geographical area but had different clinical manifestations, Kal1 was from patient suffering from severe malaria but Kal3 and Kal5 from uncomplicated malaria patients. Nucleotide blasting using NCBI database

demonstrated 76–84% identity with DBLβ domain of PfEMP-1 from distinct *P. falciparum* isolates except for Pap2 with 98% identity with PfEMP-1 of IT4/25/5 strain. The amino acid blasting also showed 96% identity with the PfEMP-1 of IT4/25/5 strain for Pap2 but a range of 44–53% identity with the PfEMP-1 proteins of several *P. falciparum* field isolates.

Our DBLβ domains except Kal2.DBLβ shared many similar features including twelve cysteine residues and blocks of highly conserved amino acids which are flanked by more extensive polymorphic regions as previously described by Smith[b] and colleagues (2000). Similar to the Smith's description concerning the consensus feature of DBLβ and C2 domain, the DBLβ domain is followed by the C2 domain which varies in length, containing four invariant cysteine residues (Figure 4.11). Twelve conserved cysteine residues in DBLβ domain are numbered as C1-C12 and the C2 domain are C13–C16. Multiple sequence alignment with other DBL domains both from PfEMP-1 DBL and EBA DBL revealed that those 12 cysteine residues correspond to C1, C4, C5, C5a, C6, C6a, C7, C7a, C8, C8a, C9 and C10, as the conserved cysteine residues are identified with an Arabic numeral and a letter to indicate their position and to maintain the original nomenclature for identifying cysteine (Table 4.6).

Tabel 4.6. Position of cysteine residues within DBLβ-C2 domain of field isolates corresponding to those of both PfEMP-1 DBL and EBA DBL.

Sequence	**Cysteine residues numbering**											
DBL domain consensus for PfEMP-1 and EBA	C1	C4	C5	C5a	C6	C6a	C7	C7a	C8	C8a	C9	C10
DBLβ domain of field isolates	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12

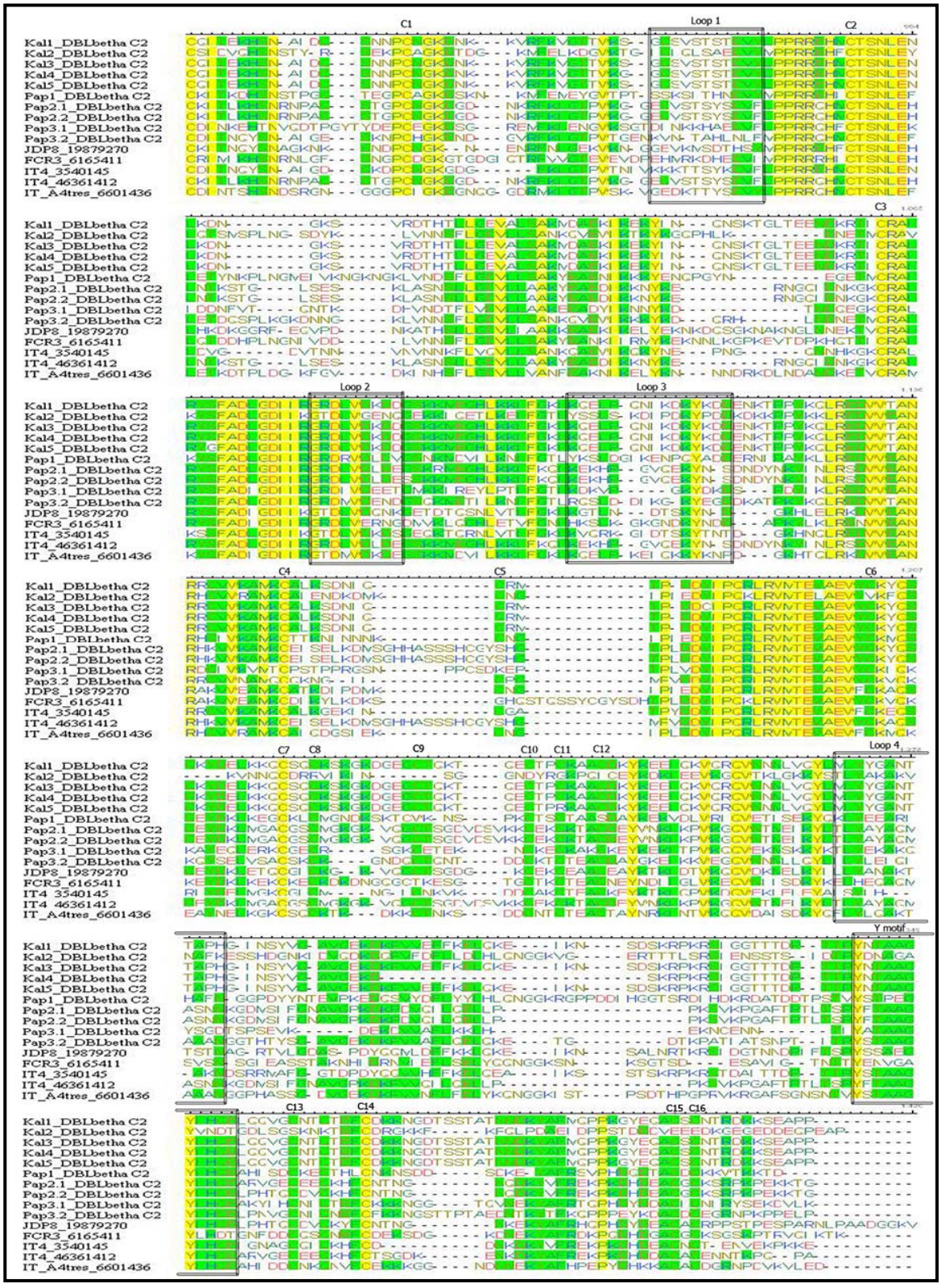


Figure 4.11. Characteristic of DBLβ-C2 sequences from field isolates. DBLβ domain of Indonesian field isolates shared many similar features including twelve cysteine residues and blocks of highly conserved amino acids flanked by more extensive polymorphic regions. The DBLβ domain is always followed by C2 domain containing four invariant cysteine residues. The conserved cysteine residues in DBLβ-C2 are numbered as C1 - C16. The four loops (loop 1-4) and 'Y motif' are indicated by boxes.

Compared to another DBLβ-C2 sequences, Kal2.DBLβ-C2 sequence presented the most different sequence feature, it lacked several conserved cysteine residues, including C6, C8-C11 of DBLβ domain and C13, C15 and C16 of C2 domain. Despite many differences with the consensus feature of DBLβ and C2 domain, Kal2.DBLβ-C2 sequence exhibited the 'Y motif' within C2 domain which was reported to play an important role in binding function of DBLβ-C2 domain. Some DBLβ-C2 sequences also revealed little differences such as Kal3 had one more cysteine residue in between C5 and C6, Kal5 lacked of C11 and was substituted by arginine (R) and Pap1 had serine (S) to substitute C12. However, all sequences (Kal3, Kal5 and Pap1) had the 'Y motif' within C2 domain (Figure 4.11).

For further confirmation, analysis by using varDom 1.0 server (http://www.cbs.dtu.dk/services/VarDom/) was conducted. It was shown that all sequences except for Kal2 were identified as DBLβ domain. Although Kal2 was not identified as DBLβ domain, it remained to be classified as Duffy binding domain (Table 4.7). HB analysis for DBLβ domain demonstrated the presence of five major HBs (HB1-5) in all sequences (Table 4.8), moreover HB53 in subdomain S1, HB146 in subdomain S2b, HB59 in subdomain S3a and HB61 and HB62 in subdomain S3b appeared as HB characteristics of DBLβ domain (Appendix 3).

Table 4.7. Sequence family classification of DBLβ-C2 domain from field isolates using varDom server

DBLβ-C2 sequence	Score for sequence family classification	
	Duffy-binding domain	DBL β domain
Pap1	345.0	719.0
Kal1	393.1	858.0
Pap2.1	384.2	855.4
Pap2.2	384.1	855.6
Kal2	255.3	-
Pap3.1	357.6	703.1
Pap3.2	368.6	823.7
Kal3	383.7	844.6
Kal4	377.3	784.7
Kal5	380.3	832.6

Table 4.8. Distribution of five major homology blocks (HB) in DBLβ domains of field isolates using VarDom server

DBLβ Sequence	Score HB4	Score HB3	Score HB5	Score HB2	Score HB1
Pap1	31.8	37.2	39.7	37.2	23.7
Pap2.1	36.6	33.4	37.8	37.2	25.5
Pap2.2	36.6	33.4	37.8	37.2	25.5
Pap3.1	31.6	34.8	36.7	37.2	33.8
Pap3.2	33.0	37.3	37.5	37.2	21.5
Kal1	34.3	37.4	45.7	37.2	23.4
Kal2	32.9	34.6	44.8	20.6	-
Kal3	34.3	37.4	45.7	31.4	23.4
Kal4	34.3	37.4	45.7	37.2	23.4
Kal5	34.3	33.4	45.7	37.2	11.3

4.9 Binding capacity of PfEMP-1 DBLβ-C2 domain to ICAM-1

Specific primers for the binding site of DBLβ-C2 domain from each sample were designed to construct recombinant plasmids expressed on the surface of mammalian COS-7 cells and tested for binding to ICAM-1. Due to previous studies which have mapped the binding residues for ICAM-1 in both DBLβ and C2 domain, specific primers for each DBLβ-C2 sequence were designed including residues of C1-C16 (3.1.12). The domain was cloned in frame with the signal sequence and the transmembrane segment of HSV gD to allow expression on the surface of transfected COS-7 cells as previously described (Chitnis and Miller, 1994). Immunofluorescence assay using a monoclonal antibody, DL6 or ID3, which is directed against HSV gD sequences in the fusion constructs (Cohen et al., 1988) were applied to confirm that all constructs tested were expressed on the surface of transfected COS-7 cells. Magnetic beads coated with ICAM-1/Fc were used for binding to transfected COS-7 cells expressing the DBLβ-C2(C1-C16) domain of each sample on their surface (Figure 4.12).

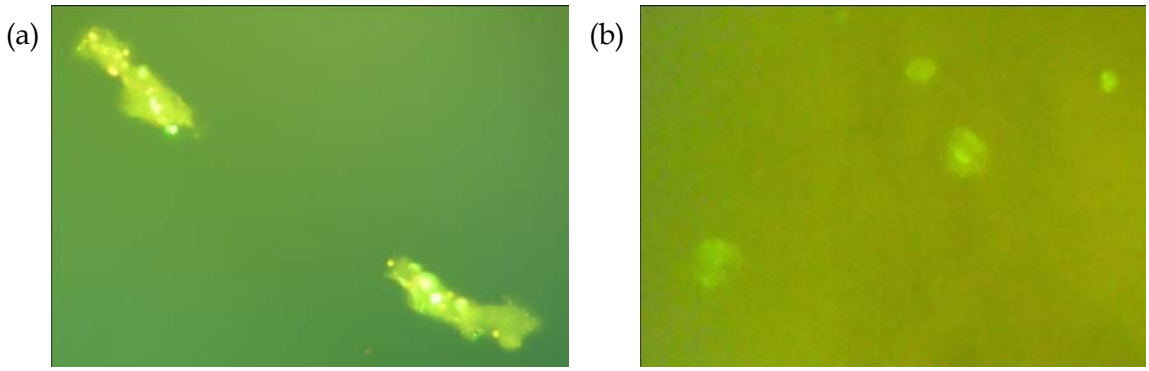


Figure 4.12. Immunofluorescence images from the staining of transfected COS-7 cells with an antibody against an epitope tag in the recombinant protein. (a) ICAM-1 coated magnetic beads bound transfected COS-7 cells expressing recombinant DBLβ-C2 of field isolates. Binding was expressed when transfected cells contained five or more ICAM-1 coated beads. (b) Transfected COS-7 cells expressing recombinant DBLβ-C2 of field isolates did not bind to ICAM-1 coated magnetic beads.

Recombinant constructs derived from samples with the clinically severe malaria presented a higher ICAM-1 binding avidity (85.0–94.5%) compared to those from uncomplicated malaria, except in one sample, Pap3 with only 1% binding avidity (Table 4.9). Interestingly, Kal3.DBLβ-C2 recombinant construct which has 99.8% and 99.4% sequence similarity with the Kal1.DBLβ-C2 and Kal5.DBLβ-C2, respectively, showed no binding avidity to ICAM-1 whereas the other two samples showed high binding avidity. Further, Kal3.DBLβ-C2 and Kal5.DBLβ-C2 which derived from samples with uncomplicated malaria demonstrated contrary binding avidity to ICAM-1. Surprisingly, Kal2.DBLβ-C2 showed high binding avidity despite the sequencing results showing low similarity with the other sequences and DBLβ-C2 in general. The mock-treated beads defined as magnetic beads without ICAM-1/Fc coating and untransfected COS-7 cells were used as negative controls for binding to ICAM-1.

Table 4.9. Binding capacity of DBLβ-C2 domain to ICAM-1

Sample	Clinical	Binding to ICAM-1 (%)[a]
Pap1.DBLβ-C2	Severe malaria	94.5
Kal1.DBLβ-C2	Severe malaria	89.0
Pap2.DBLβ-C2	Severe malaria	80.0
Kal2.DBLβ-C2	Severe malaria	85.0
Pap3.DBLβ-C2	Severe malaria	1.0
Kal3.DBLβ-C2	Uncomplicated malaria	0
Kal4.DBLβ-C2	Uncomplicated malaria	0
Kal5.DBLβ-C2	Uncomplicated malaria	88.4
Mock-treated beads[b]	-	0
Untransfected cells	-	0

[a] DBLβ-C2 recombinant proteins were expressed at the surface of COS-7 cells and tested for binding to ICAM-1/Fc coated beads. Data shown is a percentage of transfected COS-7 cells containing five or more ICAM-1 coated beads in two independent experiments, with a minimum of 50 transfected cells were analyzed for each construct.
[b] Binding was controlled with mock-treated beads i.e. beads without ICAM-1/Fc coating.

5. DISCUSSION

5.1 Human *Plasmodium knowlesi* cases

The detection of *P. knowlesi* in this study broadens the geographic distribution of natural *P. knowlesi* infection in humans. Although this is not surprising as there were previously many human *P. knowlesi* infections reported in the Malaysian part of Borneo. All four *P. knowlesi* infections were microscopically misdiagnosed as *P. falciparum*, as the early ring stage of *P. knowlesi* is morphologically similar with the early ring stage of *P. falciparum.* There was also a mixed infection with *P. falciparum* in three samples.

By using Pmk8 and Pmkr9, the sequencing results confirmed solely one out of four positive samples by the PCR. A similar result was reported from Vietnam where only two out of five positive samples with *P. knowlesi* by PCR were positive confirmed by sequencing. The Pmk8 and Pmkr9 primers target the ssrRNA-S gene expressed during the sexual stage. The sequence of the reverse primer Pmkr9 is found in *P. vivax* S-type strains and other Plasmodium spp., especially related to *P. vivax*. The forward primer Pmk8 on the other hand seemed to be more specific. Imwong and colleagues found that these *P. knowlesi* primers (Pmk8 and Pmkr9) stochastically cross-react with *P. vivax* genomic DNA resulting in false positive amplification when there is *P. vivax* genomic DNA in the sample. All samples in this study were mixed infections with *P. vivax*, as molecularly confirmed by PCR. Based on the results, it is recommended to confirm by sequencing especially in cases of mixed infection. Furthermore, sequencing helps to detect genetic variation of different Plasmodia species and populations from distinct areas.

The patient infected with *P. knowlesi* had clinically mild malaria and was successfully treated with chloroquine-primaquine. This is similar to the report by Putaporntip and colleagues that all *P. knowlesi*-infected patients in Thailand had a low parasite density and responded well to antimalarial drugs for either *P. vivax* (chloroquine-primaquine) or *P. falciparum* (mefloquine-artesunate). However, Cox-Singh and colleagues reported four fatal cases of *P. knowlesi* infection in Sarawak. All patients were relatively old (39-69 years old), presented with hyperparasitaemia and developed marked hepatorenal dysfunctions defined as signs of severe *P. falciparum* malaria. It has been suggested that the high parasite load and the compounding effect of 24 hours asexual replication cycle lead to the fatal complication in *P. knowlesi* infection. A previous study demonstrated that *P. knowlesi* is able to loose or increase its virulence during blood passages in human.

Strain differences could also occur in wild populations and might effect human differently (Coatney, 1971), resulting in a range of clinical manifestation from uncomplicated to potentially life-threatening malaria.

Regarding the large distribution of the vector and the natural host of *P. knowlesi*, it is very likely that *P. knowlesi* will be found in other parts of Indonesia. Since *P. knowlesi* is only reliably diagnosable by molecular methods, further molecular and clinical studies should be encouraged and new specific primer based on Indonesian *P. knowlesi* isolates designed.

5.2 Characteristic of malaria patients

Malaria presents with a broad range of clinical manifestations from asymptomatic to severe malaria leading to death. Once malaria takes a severe course, multiple organs are affected. Our severe malaria patients were adult and had cerebral malaria along with one or more of WHO-defined severe clinical manifestations including severe anaemia, respiratory distress, renal failure, hepatic dysfunction and jaundice. The patients features were similar to a large study in South-East Asia with predominantly adult patients and strictly defined severe malaria where 54% of the 1050 patients had cerebral malaria, 37.1% renal impairment, 50.4% jaundice, 34.4% respiratory distress, 59.4% metabolic acidosis and 18.4% severe anaemia (Dondorp et al., 2008). There are distinct patterns of severe malaria according to malaria transmission intensity. In sub-Saharan Africa where the transmission intensity tends to be high and stable, severe malaria is mainly a disease for children under 5 years with cerebral malaria, severe anaemia or respiratory distress (Marsh et al., 1995). Also in areas with moderate transmission, cerebral malaria in young children is the most common presentation (Reybun et al., 2005; Snow et al., 1994). Conversely, in areas with low transmission, such as South-East Asia, severe malaria affects all age groups, but young adults are the most affected and commonly present with multiple organ failure including cerebral malaria, renal failure, severe jaundice and pulmonary oedema (White, 1987). Further, a study from a low transmission area in Asia showed that age has a large effect on presented syndromes, with anaemia, respiratory distress and convulsion being more common in children, whereas renal and hepatic failures are more often found in adults (Dondorp, et al., 2008).

A number of age-specific rates of morbidity and mortality from epidemic falciparum malaria in Asia have been described, adults seem consistently to be at higher risk of severe disease (Dondorp et al., 2008; Mohanty et al., 2003; Greenberg and Lobel, 1990; Baird et al., 1998; Schwartz et al., 2001). Our severe patients were people from Java who

are at little or no risk of malaria infection but they moved to either Papua or South Kalimantan where malaria endemicity is higher. Those severe patients were considered to be first exposed to malaria infection. Baird[a] and colleague (1998) reported an increased risk of severe disease following primary exposure to *P. falciparum* with age among transmigrants moving from another part of Indonesia with lower risk of malaria infection to Irian Jaya (now Indonesian Papua) with the highest malaria endemicity in Indonesia. An intrinsic age-related difference in immune function is suggested leading to differences in the balance between protective and harmful effects of host immune responses to *P. falciparum,* profoundly affecting the course of infection.

Contrasting with most of our severe patients, the uncomplicated malaria patients were residents of malaria endemic area. In an area with high transmission intensity, adults are less affected to infection due to naturally acquired immunity that was developed during the long-lived exposure to the parasites. Baird (1995) hypothesised that in malaria endemic areas, naturally acquired immunity has dual aspects; an intermediate phase referred as anti-disease or anti-toxic immunity which appears in young children exposed to heavy infection, where the infected individual showing minimal or no signs of clinical malaria even in the presence of high parasitaemia, and a true anti-parasite immunity occurs in adults which preclude high-density parasitaemia and attendant disease. All of our uncomplicated cases presented with mild malaria and low parasitaemia level, indicating an existence of anti-parasite immunity.

5.3 Genotyping results

It was shown that infections in humans are frequently composed of a complex mixture of parasite clones with different genetic and phenotypic characteristics (Ntoumi et al., 1995; Conway and McBride, 1991; Greenwood et al., 1987). The number and relative ratio of the various clones present at any time point vary substantially from individual to individual. Analysis of the genetic characteristics of parasite populations using PCR und subsequent RFLP provide the advantage of sensitivity and certain specificity. The single copy genes MSP-1, MSP-2 and GLURP present large allelic polymorphisms, allowing easy detection of parasite diversity. However, the approach using PCR commonly underestimates the actual number of distinct alleles presents in the peripheral circulation at the time of sampling due to some reasons: (1) distinct parasite clones carrying the same allelic form are not differentiated, (2) alleles with identical size may present sequence differences that are not distinguished (Miller et al., 1993), (3) alleles present at very low density in a complex blood infection remain undetected (Kiwanuka, 2009).

Genotyping resulted in multiple infections per patient shown as multiplicity of infection (MOI). The MOI ranged from two to ten in each isolate, whereas samples from severe patients had higher MOI than those from uncomplicated cases. The result was consistent with a case series reported from India: mild cases had fewer clones compared to severe cases (Ranjit et al., 2004; Ranjit et al., 2005). However, other data on the association of MOI with disease severity showed conflicting results. Contrasting with our finding, studies in Senegal (Robert et al., 1996) and Nigeria (Amodu et al., 2008) reported lower MOI in severe malaria than in mild cases. But, reports from Madagascar (Durand et al., 2008), Gabon (Mayengue et al., 2007) and Sudan (A-Elbasit et al., 2007) found no association between the MOI and severity of malaria. The variability of the results between studies may be in part due to differences in transmission levels according to study sites, differences in genotyping methods and/or interpretation of data and differences in sensitivity and accuracy used in different laboratories (Faernert et al., 2001; Felger et al., 1999; Smith et al., 1999). One potential explanation for our results could be that the higher the MOI the higher is the possibility of developing severe disease. The probability of being infected with a drug-resistant strain, a more pathogenic strain or an unknown strain to the host's immune systeme is rising with rising MOI. Finally, it should be noted that malaria outcome is certainly not only determined by parasite characteristics, also host characteristics are involved. Several factors have been suggested influence the MOI including the age of the host (Ntoumi et al., 1995), human genetic polymorphisms (Ntoumi et al., 1997) and the levels of malaria endemicity and transmission intensity (Arnot, 1998).

The study only focused on the association of MOI with levels of malaria endemicity and transmission intensity. As predicted, samples from Papua had a higher MOI than those from South Kalimantan. Papua province is a malaria stable transmission area and has high endemicity class with $PfPR_{2-10} \geq 40\%$ (Elyazar et al., 2011). It has high malaria transmission intensity where individuals receive more than one infection per year (Karyana et al., 2008). In contrast, South Kalimantan province is a malaria stable transmission area with moderate endemicity class, $5\% < PfPR_{2-10} < 40\%$ (Elyazar et al., 2011). Some studies reporting the relationship between the mean number of genotypes per infected person and transmission intensity suggested that increase in transmission level is generally associated with progressive increase in the average number of malaria parasite clones per host (Appawu et al., 2004). This indicates that in areas with lower transmission intensity infected persons should significantly have fewer parasite clones (Paul et al., 1998; Paul et al., 1995). Furthermore, the antigenic and genetic repertoire of

P. falciparum populations is wider in high-transmission areas (Bendixen et al., 2001; Hoffman et al., 2001; Faernert et al., 2009). Due to crossing and random mating between gametes belonging to different parasite clones, intragenic recombination and independent chromosome assortments during meiosis in the mosquito occur more frequently (Babiker et al., 1994). This has important consequences for the global spread of multigenic phenotypes such as vector competence, parasite virulence and anti-malarial drug resistance (Paul et al., 1995).

5.4 *var* gene diversity

var gene diversity was determined by DBL1α region using αAF and αBR primers. Although the *var* gene sequences have an extensively diverse organisation, the majority of *var* genes contain the DBL1α region. The existence of homology blocks within this region has enabled researchers to design primers that can be used to amplify a DBL1α domain from the majority of samples (Taylor[a] et al., 2000) including field isolates (Bull et al., 2007).

In this study, the DBL1α region of *P. falciparum* field isolates derived from severe and uncomplicated malaria patients from two different malaria endemic areas in Indonesia was analysed. Consistent with previous studies on *var* gene diversity (Smith et al., 1995; Smith[b] et al., 2000; Taylor[a] et al., 2000; Ward et al., 1999), our results demonstrated similar variability of DBL1α sequences within and between isolates. This implies that the level of diversity represents the overall DBL1α heterogeneity that exists within each isolate.

Barry and colleagues reported the global diversity in *P. falciparum var* genes from different geographic regions. The diverse repertoire showed spatial structuring but minimal overlap in the *var* gene repertoire among isolates from different regions, indicating a global distribution of patterns (Barry et al., 2007). Our data demonstrated little overlap of field isolates from geographically close regions; only four sequences were shared each in two different isolates from the same geographical area. Comparison with published sequences also presented minimal overlap among DBL1α sequences from more different origins, concurred with previous studies that genetically diverse parasites contained essentially non-overlapping *var* gene repertoires (Freitas-Junior et al., 2000), indicating that *var* gene diversity on a global scale is immense.

*V*ar sequence diversity is generated by frequent recombinations and rearrangements within or between *var* genes, specifically through either reciprocal recombination or gene

conversion (Taylor[b] et al; 2000; Freitas-Junior et al., 2000; Kyes et al., 2007). The frequency of recombination between *var* genes depends upon chromosomal location, gene orientation and homology in the gene flanking sequence (Ups). *Var* gene recombination preferentially occurs within rather than between *var* groups, with the exception of the semi-conserved *var* homologs (*var*1CSA, *var*2CSA and type 3 *var*) that appear to recombine on their own (Kraemer et al., 2007). Further, recombination processes can occur within each parasite genome as well as between two parasite genomes during both meiosis (Freitas-Junior et al, 2000; Taylor[b] et al; 2000) and mitosis (Duffy et al., 2009). This recombination hierarchy is globally maintaining the nature of the distinct *var* gene groups.

However, selection is suggested to have a strong role in conserving *var* gene organisation, indicated by appearance of common sequences among global isolates. As *var* genes encode for variant target immunity and cytoadherence activity, the selective forces to maintain both functions have been manifested in an apparent genetic grouping of binding phenotypes to particular *var* gene sequences (Roberts et al., 1993).

5.5 Distribution motif in DBL1α sequences from genomic DNA

The DBL1α sequence tag was classified by using the cysteine/PoLV sequence grouping and the new homology block defined by Rask et al (2010). The distribution of sequence groups and HBs among isolates causing severe and uncomplicated malaria was similar, indicating that DBL1α shares common sequences among different clinical categories. According to the cysteine/PoLV sequence classification, the majority of sequences from both clinical categories belonged to sequence group 4 having four cysteine residues (cys4), referred as normal number of cysteine in DBL1α. Bull and colleagues (2007) demonstrated that the majority of group B *var* genes, which are the most frequent members of *var* genes, of group B/C and group C in laboratory isolates 3D7, HB3 and IT4 belonged to sequence group 4. Almost all group A *var* genes lacking two cysteine residues (cys2) are classified into sequence group 1-3, while sequence group 1 is exclusively found in group A *var* gene and approximately 60% of group A *var* gene belongs to sequence group 3, none of the cys2 type was found in group C *var* gene (Bull et al., 2005). Interestingly, we observed that group 3 sequences were only found in isolates having caused severe malaria. Although, we focused on DBL1α from genomic DNA, the result still provides information on the potential expression of *var* genes, where the parasite express a different *var* gene in every cycle through the switching

mechanism. Furthermore, studies on the association of expressed *var* gene with severe malaria reported a tendency of DBL1α with reduced cysteine residues to be expressed in severe malaria (Kirchgatter et al., 2002; Kyriacou et al., 2006).

Since we used the field isolates originated from two distinct geographical areas, we further analyzed the distribution motif of DBL1α sequence tag in association with the geographical origin. Concurred with a previous study which reported a similar distribution of relative number of genomic sequences in each sequence group between field isolates and laboratory isolate 3D7 (Bull et al., 2005), our result revealed a similar sequence distribution between isolates, suggesting that although extremely diverse, DBL1α shares common feature among different geographical origins.

By analyzing DBL1α sequence tags from three different continents, Bull et al. (2007) demonstrated an association of specific features i.e. MFK* at PoLV1 (group 1) or the *REY at PoLV2 (group 2 and 5) with short sequences. Our result showed similar associations, indicating that the same structural features in DBL1α sequences are shared.

Homology block (HB) analysis by varDom server (Rask et al., 2010) showed that our DBL1α sequences stretch from the most prevalent HB3 to HB2. Unfortunately, we could not detect the HB17 and HB19 as HB characteristic for DBL1α due to the length of our sequence, since HB17 and HB19 lie upstream of HB4. The difference between cys4 and cys2 could only be detected by HB36 that was present in all cys4 but absent in all cys2 sequences, as described previously (Rask et al., 2010). The presence of HB79 and HB88 in some cys4 sequences and absence in cys2 raising the possibility of those HBs for differentiating cys4 from cys2.

5.6 Expressed *var* genes from clinical field isolates

Besides using genomic DNA, *var* sequence diversity was also analysed using RNA transcripts extracted from dried blood on filter paper. This showed the expression of *var* genotypes at the time of symptoms. cDNA sequences from all severe cases but solely one of uncomplicated case could be generated. Interestingly, Maeno and colleagues (2008) using transcripts from dried blood on filter paper reported that the positivity rate of transcripts from asymptomatic patients was lower than from symptomatic cases.

Regular PCR is a stochastic process and primer bias is a major source of error in semi-quantitative expression trials. Even after repeated several times, amplification of cDNA from these two uncomplicated cases resulted in reproducible sequences with less than 12% similarity with DBLα and only short sequences matched with hypothetical or

conserved proteins from Plasmodium. Although the universal primers αAF and αBR have shown to have only a small degree of bias in their specificity and possess a wide range in targeting *var* gene sequences, we could not excluded a primer bias as a cause of the undesired result.

Previous studies showed contradictory data on the timing of *var* gene transcription, reverse transcription-polymerase chain reaction (RT-PCR) data suggested that many *var* gene types are transcribed in early ring stages and exclusively one major PfEMP-1-encoding type is transcribed in later pigmented trophozoite stages (Chen et al., 1998; Scherf et al., 1998), whereas northern blot data showed that almost all full-length *var* transcripts encoding PfEMP-1 type were detected only in ring stages (Keys et al., 2003). Others argue that a single full-length *var* gene is transcribed both in ring and trophozoite stages in clonal parasites (Dahlback et al., 2007; Duffy et al., 2002; Noviyanti et al., 2001; Peters et al., 2002). However, most agree that only one *var* gene is dominantly expressed as a single PfEMP-1 in the late ring stage and mature trophozoite stage of parasite. The presence of a dominant transcript is proposed due to higher level of that transcript and rapid decay of low-level, spurious short- and sometimes full-length transcripts (Rowe et al., 1997; Mok et al., 2007; Chen et al., 1998).

A study argued that RT-PCR of ring stages, as we did in this study, is the wrong method to find the major *var* gene transcript present in a given parasite sample. In most cases, mature forms of the parasite are not found in the peripheral circulation during infection due to adhesion of the IEs to microvascular endothelium and sequestration in variety of organs (Miller et al., 2002). This is the major question in studies of expression trials with field isolates if the parasites in the peripheral blood adequately represent the sequestered population. Ideally, studies addressing the relationship between *var* gene expression and specific disease syndromes have to examine sequestered parasites, but these are only accessible in post-mortem samples. Two studies indicated that the dominant parasite genotype of sequestered parasites is usually the same as in the peripheral blood (Dembo et al., 2006) and that the parasite genotype in cerebral malaria patients is homogeneously distributed throughout the body (Montgomery et al., 2006). In addition, by performing agglutination assays, Bull et al. (1998) observed a distinct parasite subset in peripheral blood of children with severe malaria compared to children with mild malaria, indicating the presence of virulent parasites in the periphery. Although these studies do not explain if the *var* gene profile of the peripheral blood parasites reflects that of sequestered population, they are consistent with the possibility that the peripheral blood population could adequately reflect the sequestered parasite. However, theoretically the

ring stages have a higher relative level of the relevant PfEMP-1-encoding *var* transcripts, but practically in several studies showed that pigmented trophozoites yield more RNA, so both stages should be valid for investigating repertoires of *var* transcription by RT-PCR (Kyes et al., 2003).

Observation of distribution motif in DBL1α showed a lack of two cysteine residues (cys2) in all DBL1α expressed sequences from patients. It is likely that sequences lacking two cysteine residues (cys2) are more frequently expressed than those with a normal number of cysteine (cys4). All DBL1α expressed sequences from blood of severe patients lacked of two cysteine residues. Unfortunately, sample size was too low to compare the DBL1α expression with those from uncomplicated patients. However, some studies comparing the expression between severe and uncomplicated case reported the tendency of cys2 DBL1α to be expressed in severe patients (Kirchgatter and del Potillo, 2002; Bull et al., 2005; Kyriacou et al., 2006; Kraemer et al., 2007).

Several studies about a possible association of either *var* gene grouping or DBL1α motifs with clinical malaria manifestations showed conflicting results. A study in Malian children with cerebral malaria showed predominantly expressed cys2 (referred as DBL1α1 type), characteristic of group A or B/A *var* gene, whereas a non-virulent form with hyperparasitaemia showed predominantly expressed cys4 (referred as DBL1α0 type), characteristic of group B or C *var* gene (Kyriacou et al., 2006). Another study using a selected 3D7 clone reported an association of up-regulation of group A *var* gene and expression, which can be detected on the surface of IE carrying PfEMP-1 type, with severe malaria (Jensen et al., 2004). Conversely, a study in Papua New Guinea showed up-regulation of group B *var* gene in clinical disease (severe and mild) compared to asymptomatic infection with predominantly expressed group C *var* gene (Kaestli et al., 2006), but it should be noted that the severe disease in this study included a variety of clinical syndromes and not strictly defined cerebral malaria. A study from Tanzania also found an increased expression of both group A and B *var* genes in severe compared to uncomplicated and asymptomatic patients (Rottmann et al., 2006). Another study in Kenya did not find any association between expression of specific *var* gene groups and disease manifestations (Bull et al., 2005). In this study, we did not directly detect the upstream sequence (Ups) of expressed sequences to differentiate the *var* gene group, but blasting results revealed that all expressed sequences had identity of 82-99% with the UpsA *var* gene from distinct isolates, indicating group A *var* gene expression in all of our samples (4 severe and 1 uncomplicated case).

Investigation of DBL1α sequence from one synchronised *P. falciparum* culture showed that both cys2 and cys4 sequences were expressed during trophozoite stage. Although a study reported that trophozoite-parasitized erythrocytes could be the best source of RNA to determine the dominantly transcribed and translated *var* gene (Blomqvist et al., 2010), *var* gene expression changes may occur early in *in vitro* adapted cultures (Peters et al., 2007; Southwell et al., 1989). Therefore, one can assume that results from the cultured parasites might not represent the expression pattern in the patient.

5.7 Conserved DBL domains

To determine the association of specific sequences and clinical outcome of malaria, first genomic DNA and cDNA was amplified with UNIEBP primers, and an internal specific primer based on the amplified fragment was designed. The degenerated UNIEBP primers were designed from conserved amino acid sequences PRRQKLC (Homology block 'B' by Smith) and PQFLRW (Homology Block 'H' by Smith) in the DBL domain to allow amplification of expressed DBL sequences. As predicted, multiple bands were generated indicating the diversity of the parasite lines in each sample, as confirmed by genotyping results. The number of observed bands probably underestimates the number of amplifiable sequences due to overlapping of same size-products and the possibility of preferential amplification of some sequences over others (Peterson et al., 1995).

The amplified sequences from the transcripts varied in number and length and were identified as transcripts from the single copy gene *eba-175* and from the *var* gene family. As described previously (Peterson et al. 1995), both genes contain an adhesive DBL domain. The *eba-175* gene encodes for the erythrocytes binding antigen (EBA)-175 protein associated with erythrocyte invasion and the multi-copy *var* gene family encodes for PfEMP-1 served as cytoadherence ligand and mediated cytoadhesion and sequestration. Both proteins play important roles during the blood stage infection (Howell et al., 2006; Michon et al., 2002). It is proposed that DBL domains of PfEMP-1 evolved from EBAs, because erythrocyte invasion is an essential property for survival and transmission of all *Plasmodium* species and cytoadherence is a restricted property of a subset of *Plasmodium* species such as *P. falciparum*, and *P. reichenowi* (Smith[b] et al., 2000).

One of the amplified transcripts was a 486 bp band that matched well with sequences from DBLγ domain of *var*1CSA 3D7 and DBLγ domain of placenta from Malawian women with PAM. It can be assumed that both sequences correspond to each other as

placental isolates commonly express DBLγ containing subtypes of *var* genes with homology to either 3D7*var*5.2 (var_{COMMON}) or FCR3*var*CSA (Winter et al., 2003). Though, this 486 band was only found in a male patient from Papua (Pap3). The sequence is probably a *var*1CSA-type gene (also defined as var_{COMMON}). *var*1CSA is highly conserved between isolates, reported as constitutively transcribed in approximately 60% of malaria infected children in Gabon (Winter et al., 2003) and in non-pregnant donors (Fried[b] and Duffy, 2002), and can be detected by RT-PCR in many isolates independent from stage and binding phenotype. It is therefore not a strict characteristic of isolates from pregnant women or placental malaria (Winter et al., 2003; Kyes et al., 2003). In 3D7, *var*1CSA is present on chromosome 5 (*var*5.2), it has an UpsA and is constitutively transcribed in the opposite direction to most other *var* genes. It lacks a regulatory intron and an acidic terminal segment (exon 2) and ends in telomeric repeat sequences (Rask et al., 2010). *var*1CSA encodes a large, hypothetical PfEMP-1 of a structure similar to previous placenta-binding PfEMP-1 but it is not present at the IE-surface possibly due to an absence of a potential trans-membrane anchor sequence and the internal ATS domain. The function of *var*1CSA for the parasite remains unclear. However the fact that some isolates do not contain the *var*1CSA transcripts indicate that the gene is not essential for the parasite survival (Winter et al., 2003).

5.8 Specific motif *var* D-like sequence

In a report concerning the association of a specific genotype with severe malaria, Ariey and colleagues (2001) have designed a specific *var* D gene primer. It was based on a 520 bp band that resulted from amplification using UNIEBP primers and was observed in most of the severe patients. By using similar methods, we designed a specific primer targeting the *var* D-like gene. The amplified sequence corresponded partly to the *var* D gene reported previously (Ariey et al., 2001) but closely matched with the DBLγ domain of PfEMP-1 isolated from 3D7 and patients with PAM from various origins. According to Ariey et al, the *var* D gene possessed characteristics of the DBLδ domain, while our sequences corresponded to DBLγ domain of PfEMP-1. Further analysis found three conserved sequence motifs containing 6-10 nucleotides within all proposed *var* D genes. Analysis of homology blocks by using varDom server showed HB86 in the previously described *var* D gene and in one of our sequences which is mostly found in the subdomain S2 of DBLγ, while another *var* D-like sequences showed HB27 which is largely found in DBLδ and HB98 which is observed in some of both DBLδ and DBLγ

domains. One other sequence showed HB82 which is found in small number of DBLδ and DBLγ domains, and one showed only HB3 which is found in all DBL domains. As only HB3 to HB5 was covered, the origin of our sequences remains uncertain, either DBLγ or DBLδ is possible. Rask and colleagues discussed the reminiscences of DBLγ and DBLδ domains, which might explain the results (Rask et al., 2010).

Interestingly, Ariey and colleagues reported an absence of the gene from most laboratory strains (FCR3, Palo Alto, FCC1, Tak9/100, Tak9/96, ItG2G1), while we observed a single fragment of 240 bp from the laboratory strain K1, which has a 25,7% similarity with the *var D* gene reported by Ariey (AJ277137).

In this study, the *var D*-like was found in genomic DNA but not in RNA transcripts of uncomplicated malaria isolates. A low identity of our *var* D-like gene with previously proposed *var* D gene made it difficult to refer those sequences as the *var* D gene. However, the increased prevalence and programmed expression of those sequences containing certain HBs solely in severe malaria implies its involvement in the pathogenesis of malaria. Though, sample size in this study was too small to indicate statistically valuable information.

5.9 PfEMP-1 DBLβ-C2 domain and its ICAM-1 binding capacity

In *Plasmodium falciparum*, DBL domains are present in both EBA used during erythrocyte invasion and PfEMP-1 mediated cytoadherence. PfEMP-1 DBL domains diverged significantly from the presumed ancestral EBA DBL domains and cluster into six distinct sequence classes: α, β, γ, δ, ε, and X (Smith[b] et al., 2000). Whereas the EBA DBL domains contain 12-13 invariant cysteine residues (Adams et al., 1992), the absolute number of cysteine residues differs more significantly between PfEMP-1 DBL domains. However, homology between EBA and PfEMP-1 DBL domains ends shortly after the 10th cysteine residue and all the first 10 cysteine residues in EBA DBL can be identified in PfEMP-1 DBL domain (Smith[b] et al., 2000).

In this study, we identified the DBLβ-C2 domain of *P. falciparum* field isolates derived from patients with severe and uncomplicated malaria. Similar to the previous reports (Smith[a] et al., 2000; Chattopadhyay et al., 2004), multiple alignment analysis showed that the DBLβ sequences share many similar features, including 12 conserved cysteine residues (C1-C12) and blocks of strong amino acid conservation which are flanked by more extensive polymorphic regions, where the sequences are followed by the C2 domain which varies in length, including four invariant cysteine residues (C13-C16). The

C2 domain is also characterized by the presence of the 'Y motif' which is suggested to have an important role in binding function. The presence of conserved cysteines and homology blocks in DBLβ-C2 domain suggested conserved structural features like helices and loops, which in turn indicate a similar general architecture.

Expression of the DBLβ-C2 domain containing 16 conserved cysteine residues from different *P. falciparum* field isolates on the surface of COS-7 cells and binding assays with the ICAM-1 coated magnetic beads helped to identify the DBLβ-C2 domain as the ICAM-1 binding site. Previous studies by using the A4tres isolate (Smith[a] et al, 2000) and the JDP8 isolate (Chattopadhyay et al., 2004) showed that the ICAM-1 binding region lies in the DBLβ-C2 domain with 16 conserved cysteine residues, indicating that both DBLβ and C2 are required for ICAM-1 binding. However, not all DBLβ-C2 tandems bind ICAM-1. Recombination of DBLβ and C2 domain of ICAM-1 binding and non-binding isolates did not bind ICAM-1. Both DBLβ and C2 regions of ICAM-1 binding isolates seem to contain contact residues required for ICAM-1 binding (Mayor et al., 2005). Further, chimera containing the DBLβ and C2 domain from two different ICAM-1 binding isolates also did not bind ICAM-1, suggesting the functional interdependence between two binding domains from the same protein (Springer et al., 2004). Truncated analysis demonstrated that the critical ICAM-1 binding regions are located between a conserved tryptophan (W) and the first half of the C2 domain including the 'Y motif' (Figure 4.10). However, the optimal binding activity is dependent upon residues in the first part of DBLβ domain (Springer et al., 2004).

Multiple alignment of both ICAM-1 binding and non-binding DBLβ-C2 domains revealed that there is no common sequences shared by ICAM-1 binding DBLβ-C2 domains or absent in non-binding DBLβC2 domains. It is therefore unlikely that the ICAM-1 binding site lies in a linear sequence stretch within DBLβ-C2 domain (Chattopadhyay et al., 2004). In addition, a neighbour-joining analysis indicated the difficulties to strictly distinguish ICAM-1 binding from non-binding sequences by traditional phylogenetic criteria because ICAM-1 binding sequences did not group separately from non-binders (Howell et al., 2008).

The DBLβ-C2 domain use a common interaction site for ICAM-1 binding that maps to the equivalent glycan binding region in the EBA-175 F2 DBL domain, which consists of three distinct binding pockets referred to as glycan 5, glycan 1 and glycan 3, located at flexible loops (Tolia et al., 2005). Homology modelling also suggested that the first part

of C2 domain including 'Y motif' is actually part of the DBL domain, explaining the role of 'Y motif' in ICAM-1 binding function (Tolia et al., 2005; Singh et al., 2006).

A study using high resolution conformation model for binding DBLβ-C2 domains and ICAM-1 showed that there are four DBLβC2 loop regions contributing to the site of ICAM-1 binding (Figure 4.10): loop 1 (E21 – V30), loop 2 (G112 – E120), loop 3 (K137 – D125) and loop 4 (T283 – N293). Loop 1 is more important for the interaction between DBLβ-C2 domain and ICAM-1 and the sequences might modulate the binding affinity in different *P. falciparum* strains (Bertonati and Tramontano, 2007). Two studies observed several conserved motifs in those loops such as threonin (T) residue in loop 1, a conserved 3-amino acid motif in loop 3 (Oleinikov et al., 2009) and alanin (A) or leucine (L) in loop 4 (Bertonati and Tramontano, 2007) from ICAM-1 binding sequences. However, our results demonstrated no amino acid conservation in those loops from both ICAM-1 binding and non-binding sequences, supporting previous reports that both sequences share no common sequences (Chattopadhyay et al., 2004) Classical phylogenetic criteria were not able to differentiate both sequences indicating no possibilities to predict binding function from sequence criteria alone (Howell et al., 2008). From a perspective of protein interaction, it is known that ligand interactions are not significantly altered by substituting a single residue that participates in binding, as long as the substitution fits into the structure without clashes and does not affect structural integrity such as substitution in flexible loops (Weber, 1992). Mutagenesis study by substituting a residue in a loop supported this theory and revealed that the loss of binding to ICAM-1 is not due to one single substitution but a combinations of substitutions (Howell et al., 2008; Oleinikov et al., 2009).

Our results showed that two sequences derived from two different samples with similar clinical manifestations revealed converse ICAM-1 binding avidity although they were 99.8% identical. DBL-based primers used in this study, to obtain the DBLβ-C2 domain, only amplify a portion of the entire DBL domain. When expressed as recombinant proteins they might not fold properly and incorrect folding might affect the protein function. Moreover, since every protein is unique, false negative binding activity could not be excluded without direct proof of correct folding. We also observed that two chimeras derived from patients with different clinical malaria manifestations had a similar binding avidity to ICAM-1. But overall, DBLβ-C2 domains from severe patients showed higher binding avidity to ICAM-1 than those from uncomplicated patients. This observation is similar to previous studies (Newbold et al., 1997; Turner at al., 1994,

Turner et al., 1998) which reported the association of ICAM-1 binding capacity with disease severity. In contrast, a study in Malawian children showed that binding capacity to ICAM-1 was lowest in children with cerebral malaria (Rogerson et al., 1999). Studies in Thai field isolates found no correlation between ICAM-1 binding and the severity of disease (Udomsangpetch et al., 1996; Ockenhouse et al., 1991). However, the low affinity of most malaria parasites for ICAM-1 indicates that ICAM-1 only mediates initial rolling of IEs on the endothelial lining (Craig et al., 1997; Yipp et al., 2000). The binding to ICAM-1 is also relatively weak and can not alone trigger massive sequestration. For stable binding, other receptors such as CD36 and synergisms are most likely required (McCormick et al., 1997). Thus, resolution of the association between ICAM-1 adhesion and severe disease requires further examination of the adhesive performance of patient isolates from different clinical manifestations with a bigger sample size.

ICAM-1 is a member of the immunoglobulin superfamily and contains five Ig-like domains that are expressed on the surface of a wide range of cell types including endothelial cells. The interaction site between PfEMP-1 and ICAM-1 has been mapped to the three β strands, termed B, E and D (the BED side) of the N-terminal Ig-like domain (Berendt et al., 1992; Ockenhouse et al., 1992; Tse et al., 2004). This site is distinct, but shows some overlap with the binding site for lymphocyte function associated antigen 1 (LFA-1) and a high level of overlap with the fibrinogen binding site (Chakravorty and Craig, 2005). Although different PfEMP-1 proteins appear to bind to a similar region of ICAM-1, there are discrete variations in the specific contact residues, as it was shown to affect ICAM-1 binding affinity under flow conditions. It indicates that the ICAM-1 binding ability has originated from a common variant, and differences in binding are probably dictated by the high degree of DBLβ-C2 sequence variation in the PfEMP-1 (Cooke et al., 1994; Gray et al., 2003; Tse et al., 2004).

6. SUMMARY

Malaria is a global health problem with hundreds of million people around the world suffering from disease and estimated 1-3 millions deaths each year. There are four *Plasmodium* species causing malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*.,Recently, it has been reported that *Plasmodium knowlesi,* a monkey Plasmodium species in South-East Asian countries, causes epidemiologically relevant infections in humans, proposing *P knowlesi* as the fifth humanpathogen malaria species. Microscopically, it is difficult to differentiate *P. knowlesi* from *P. falciparum* and/or *P. malariae*.

P. falciparum causes the most severe clinical symptoms and is mainly responsible for complicated and lethal disease due to malaria. Two characteristics of *P. falciparum* seem to be relevant: (1) the capability to invade erythrocytes of all stages, thus resulting in very high parasitaemia, (2) the ability of infected erythrocytes to adhere to the vascular endothelium and other host cells (called cytoadherence). The latter might result in obstruction of the microcirculation leading to poor perfusion of host tissues, hypoxia, dysfunction of affected organs, resulting further in multiple organ failure. To avoid clearance by the human immune system, *P. falciparum* continuously changes its antigenic surface proteins which are susceptible to antibody recognition and attack. This phenomenon is called antigenic variation. One of the proteins responsible for cytoadherence and antigenic variation is *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). PfEMP-1 is a polymorphic protein that varies in domain composition and binding specificity. It is encoded by the *var* gene family consisting of ~60 variable genes per haploid genome. PfEMP-1 possesses an extra-cellular and intra-cellular part. The extra-cellular part is highly polymorphic and contains NTS followed by three distinct binding domains: DBL, CIDR and C2.

In this study, the *P. falciparum var* genes from Indonesian field isolates were characterised and the binding capacity to ICAM-1, one of the most important host receptors, were examined. One of the important questions was if any differences could be observed between isolates from patients with severe and uncomplicated malaria, according to the WHO criteria.

Twelve Indonesian malaria patients who were microscopically diagnosed as *P. falciparum,* were enrolled in this study. Due to the detection of *P. knowlesi* infections in several neighboring countries of Indonesia, all samples were examined for *P. knowlesi* by PCR. Amplification using recently published primers Pmk8 and Pmkr9 generated a

single band of 153 bp in four samples from the South Kalimantan province, the Indonesian part of Borneo. All *P. knowlesi*-positive samples were mixed infections with *P. vivax* and/or *P. falciparum* and microscopically diagnosed as mono-infection of *P. falciparum*. Direct sequencing confirmed solely one *P. knowlesi* infection out of four positive samples by PCR. At the same time, it was found that the used primers stochastically cross-react with *P. vivax* genomic DNA resulting in false positive amplification when *P. vivax* genomic DNA is present. Hence, sequencing was recommended for definite confirmation of *P. knowlesi*. Further studies on genetic variation should be encouraged in order to design new specific primers based on Indonesian *P. knowlesi* isolates.

For further investigations, 8 Indonesian samples with *P. falciparum* mono-infection were included: 5 with severe and 3 with uncomplicated malaria. First, the diversity of *P. falciparum var* genes was examined by using universal primers. To amplify the majority of the *var* gene repertoire, primers were used binding to highly conserved regions in DBL1α that are present in most of the *var* gene family members. We identified seventy-one different sequences out of 104 DBL1α sequenced clones, resulting in an average of 8.9 different DBL1α sequences per isolate. The average DBL1α sequence similarity within isolates was as high as among isolates, implying the overall DBL1α heterogeneity that exists within each isolate. Little overlap of field isolates from geographically close regions and among DBL1α sequences from more different origins occurred, indicating that *var* gene diversity on a global scale is immense. Phylogenetic analysis demonstrated no clustering of sequences regarding strain or geographical origin.

In order to identify an association of restricted motifs in DBL1α domain with malaria clinical outcome, the DBL1α sequence was analysed by distribution of semi-conserved features (cysteine/PoLV1-4 grouping) and classified into six sequence groups. Overall, there was no evidence for a difference between DBL1α sequence motifs between severe and uncomplicated malaria ($p=0.48$), indicating common sequences in DBL1α are shared among different clinical categories. Interestingly, all sequences of group 3 containing cys2 (meaning DBL1α lacked two cysteine residues) were obtained from severe cases.

Distribution of motifs in DBL1α showed cys2 type in all expressed sequences *in vivo* and in two third of expressed sequences from culture, supporting previous reports that cys2 sequences are more frequently expressed than those with a normal number of cysteines (cys4). Some studies comparing the expression between severe and uncomplicated cases reported the propensity of cys2 DBL1α to be expressed in severe malaria patients. All

DBL1α expressed sequences from blood of severely ill patients showed cys2 but sample size was to small for comparison of the DBL1α expression in uncomplicated and severe malaria.

Ariey and colleagues proposed a specific genotype called *var* D gene to be associated with severe malaria. The proposed *var* D gene matched with the DBLδ domain. By using a similar method, we designed a specific primer, targeting the '*var* D-like' gene. Amplification yielded multiple fragments from genomic DNA of all samples, but generated a single ~237 bp fragment of expressed sequences from four severe malaria patients only. Sequences highly (80-94% identity) matched with the DBLγ domain of PfEMP-1 from several isolates, sequence identity to the proposed *var* D gene was 29.0, 28, 27 and 19% for *var* D-like sequence of Kal2, Pap3, Pap1 and Pap2, respectively. Interestingly, analysis by using varDom server presented homology block (HB) characteristics of DBLγ and DBLδ. Prevalence and expression of those sequences solely in severe malaria implies potential involvement in the pathogenesis of malaria. However, sample size in this study was too small to indicate statistically valuable information.

To further analyse the *var* gene and PfEMP-1, we explored the DBLβ domain. This domain, in tandem with C2 domain binds ICAM-1 in some *P. falciparum* isolates. In this study, the *P. falciparum* DBLβ-C2 sequence from Indonesian's field isolates was amplified. Specific primers for the DBLβ-C2 sequence were designed according to several referred sequences of the DBLβ-C2 domain. The amplified ~1.7 kb band was cloned and sequenced. One to two different sequences in each sample were identified, but three sequences from distinct samples of the same geographical origin presented more than 99% sequence similarity. Blasting results corresponded to the DBLβ domain of PfEMP-1 from various *P. falciparum* isolates. The DBLβ sequences shared many similar features, including 12 conserved cysteine residues (C1-C12) and blocks of amino acid conservations which are flanked by polymorphic regions. The DBLβ domain is followed by the C2 domain that varies in length, including four invariant cysteine residues (C13-C16). The C2 domain is also characterized by the presence of the 'Y motif' which is suggested to have an important role in binding function.

The DBLβ-C2 domain with 16 conserved cysteine residues from eight *P. falciparum* field isolates was expressed on the surface of COS-7 cells for binding assays with the ICAM-1 coated magnetic beads. Recombinant constructs derived from samples with clinically severe malaria presented a higher ICAM-1 binding avidity compared to those from

uncomplicated malaria, supporting previous reports on the association of ICAM-1 with severe malaria. However, resolution of the association between ICAM-1 adhesion and severe disease requires further examination of the adhesive performance. Isolates from patients with different clinical manifestations and much larger sample size are needed.

7. ZUSAMMENFASSUNG

Malaria ist ein globales Gesundheitsproblem mit mehreren hundert Millionen Menschen auf der ganzen Welt, die jedes Jahr unter der Krankheit leiden, ca 1-3 Millionen der Erkrankten versterben. Es gibt vier verschiedene Malariaerreger des Menschen: *Plasmodium falciparum, P. vivax, P. ovale* und *P. malariae.* Erst vor kurzem wurde bekannt, dass *P. knowlesi*, eine bei Makakken verbreitete Plasmodien Spezies, in Südostasien zu epidemiologisch relevanten Infektionen und Erkrankungen beim Menschen führt und daher mittlerweile auch als fünfter humanpathogener Malariaerreger bezeichnet wird. Mikroskopisch ist *P. knowlesi* nicht sicher von *P. falciparum* und/oder *P. malariae* zu unterscheiden.

P. falciparum verursacht die bei weitem schwersten Krankheitsverläufe und ist ganz überwiegend für komplizierte Verläufe und Todesfälle verantwortlich. Hierfür scheinen vor allem zwei Eigenschaften von *P. falciparum* bedeutsam zu sein: (1) die Fähigkeit der Erreger alle Erythrozyten zu parasitieren und damit hohe Parasitämien verursachen zu können, (2) die Bindung parasitierter Erythrozyten an Gefässendothelien und andere Wirtszellen (Zytoadhärenz). Letzteres kann zur Obstruktion der Mikrozirkulation und damit zu verminderter Organperfusion mit Hypoxie und Gewebsschädigung und schliesslich zum Multiorganversagen führen. Um der menschlichen Immunabwehr zu entgehen, ändert *P. falciparum* kontinuierlich seine Oberflächenantigene, die anfällig für Antikörper-Erkennung und -angriff sind. Dieses Phänomen wird antigenische Variation genannt. Eines der Proteine für Zytoadhärenz und antigenische Variation bei *P. falciparum* ist das Erythrozytenmembranprotein-1 (PfEMP-1). PfEMP-1 ist polymorph und variiert in Zusammensetzung und Bindungsspezifität. Es wird von der *var*-Gen-Familie, bestehend aus ~60 variablen Genen pro haploidem Genom, kodiert. PfEMP-1 besitzt einen extrazellulären und einen intrazellulären Teil. Letzterer ist sehr polymorph und enthält drei verschiedene Domänen: DBL, CIDR und C2.

In der vorliegenden Arbeit sollten die *var*-Gene von *P. falciparum* Patientenisolaten aus Indonesien charakterisiert werden und deren Bindungskapazität an ICAM-1, einem der wichtigsten Wirtsliganden der Zytoadhärenz untersucht werden. Eine der primären Fragestellungen war, ob es hierbei Unterschiede zwischen Isolaten von Patienten gibt, bei denen entsprechend den WHO-Kriterien eine komplizierte oder eine unkomplizierter Malaria vorlag.

Insgesamt standen 12 verschiedene Isolate von indonesischen Patienten zur Verfügung, die mikroskopisch als *P. falciparum* diagnostiziert worden waren. Aufgrund des Nachweises von *P. knowlesi* in mehreren indonesischen Nachbarländern wurden alle Proben zunächst mittels PCR auf *P. knowlesi* untersucht. Die Amplifikation mittels bereits veröffentlichter Primer (Pmk8 und Pmkr9) generierte eine einzelne Bande von 153 bp in vier Proben aus der Süd-Kalimantan Provinz, dem indonesischen Teil von Borneo. Alle *P. knowlesi*-positiven Proben waren Mischinfektionen mit *P. vivax* und/oder *P. falciparum* und mikroskopisch als Mono-Infektion mit *P. falciparum* diagnostiziert worden. Die direkte Sequenzierung bestätigt lediglich eine *P. knowlesi* Infektion von vier PCR-positiven Proben. Gleichzeitig wurde festgestellt, dass die verwendeten Primer mit genomischer DNA von *P. vivax* stochastisch kreuzreagieren, was zu falsch positiven Ergebnissen führte. Zur sicheren Bestätigung einer *P. knowlesi* Infektion ist daher eine Sequenzierung empfehlenswert sowie weitergehende Untersuchungen zur genetischen Variation der Spezies und zum Design neuer spezifischer Primer für indonesische *P. knowlesi* Isolate.

Für die weiteren Untersuchungen standen somit 8 indonesische Feldisolate mit *P. falciparum*-Monoinfektionen zur Verfügung; 5 mit komplizierter und 3 mit unkomplizierter Erkrankung. Zunächst wurde die Diversität von *P. falciparum var*-Genen mittels universeller Primer untersucht. Um die Mehrheit des *var* Gen-Repertoires zu amplifizieren, werden Primer verwendet, die an hoch konservierte Regionen der DBL1α-Domäne binden, welche in den meisten *var* Genen gefunden wird. Es konnten hierbei 71 verschiedene Sequenzen von 104 sequenzierten Klonen von DBL1α bei durchschnittlich 8,9 unterschiedlichen DBL1α Sequenzen pro Isolat identifiziert werden. Die durchschnittliche DBL1α Sequenz-Ähnlichkeit innerhalb der Isolate war so hoch wie die der Isolate untereinander, was für eine allgemeine DBL1α Heterogenität spricht. Es zeigte sich keine Überschneidung bei Feldisolaten von geographisch benachbarten Regionen oder bei DBL1α Sequenzen von unterschiedlicher Herkunft. Dies deutet auf eine *var* Gen-Diversität auf globaler Ebene von immensem Ausmaß. Phylogenetische Analysen zeigten kein Clustering bezüglich einzelner Parasitenstämme oder der geographischen Herkunft.

Um eine Assoziation bestimmter Motive der DBL1α-Domäne mit dem klinischen Bild der Malaria zu identifizieren, wurden die Sequenzen auf semi-konservierte Verteilungsmuster (cysteine/PoLV1-4 Gruppierung) untersucht und in sechs Sequenz-Gruppen klassifiziert. Insgesamt gab es keine Anzeichen für einen Unterschied von

DBL1α Sequenz-Motiven bei schwerer bzw. unkomplizierter Malaria (p = 0,48), die Sequenz-Gruppen von DBL1α waren auf alle klinischen Kategorien verteilt. Interessanterweise wurden alle Sequenzen der Gruppe 3 mit zwei Cysteinen in schweren Fällen gefunden (Cys2, statt vier Cysteine, Cys4, es fehlen also zwei Cystein-Reste).

Die Verteilung der Motive in DBL1α zeigte den Cys2 Typ in allen exprimierten Sequenzen *in vivo* und in zwei Drittel der exprimierten Sequenzen aus der Kultur. Dies bestätigte frühere Berichte, dass Cys2-haltige Sequenzen häufiger als Cys4-haltige exprimiert werden. Einige Studien beobachteten eine erhöhte Expression von Cys2-haltigen DBL1α Sequenzen bei schwerer Malaria im Vergleich zu leichten Fällen. Alle hier untersuchten exprimierten DBL1α Sequenzen aus dem Blut schwer erkrankter Patienten zeigte zwei Cysteinreste, aber die Stichprobengröße war zu klein für einen Vergleich der Expression von DBL1α zwischen unkomplizierter und schwerer Malaria.

Ariey und Kollegen hatten die Expression eines bestimmten Genotypes, das so genannte *var* D-Gen, in Verbindung mit schwerer Malaria gebracht. Das vorgeschlagene *var* D-Gen stimmte mit der DBLδ Domain überein. Durch die Verwendung einer ähnlichen Methode haben wir spezielle Primer für ein '*var* D-ähnliches"-Gen konstruiert. Die Amplifikation ergab mehrere Fragmente aus genomischer DNA von allen Proben, erzeugte jedoch nur ein einzelnes Fragment von ca 237 Basenpaaren aus RNA von vier Patienten mit schwerer Malaria. Die Sequenzen zeigte große Ähnlichkeit (80-94%) mit der DBLγ Domain von PfEMP-1 unterschiedlicher Isolate. Die Sequenz-Identität mit dem vorgeschlagenen *var*-D-Gen betrug jedoch nur 29, 28, 27 und 19% für die jeweiligen Isolate. Eine Analyse unter Verwendung des varDom Servers zeigte interessanterweise Homologie-Block (HB) Charakteristiken von DBLγ und DBLδ. Die Expression dieser Sequenzen nur bei schwerer Malaria impliziert eine potentielle Beteiligung an der Pathogenese der Malaria. Allerdings war die Stichprobengröße in dieser Studie zu klein für eine statistisch valide Aussage.

Zur weiteren Analyse des *var*-Gens und PfEMP-1 wurde die DBLβ Domäne untersucht. Diese Domäne kann im Tandem mit der C2-Domäne ICAM-1 binden. In dieser Studie wurde die *P. falciparum* DBLβ-C2-Sequenz aus den indonesischen Feldisolaten amplifiziert. Spezifische Primer für die DBLβ-C2-Sequenz wurden für die DBLβ-C2-Domäne konstruiert und die ~1,7 kb-schwere amplifizierte Bande kloniert und sequenziert. Ein bis zwei unterschiedliche Sequenzen wurden in jeder Probe identifiziert, drei Sequenzen aus unterschiedlichen Proben derselben geographischen

Herkunft zeigten mehr als 99% Übereinstimmung. Die Sequenzen korrespondierten mit der DBLβ Domäne von PfEMP-1 verschiedener *P. falciparum*-Isolate. Die DBLβ Sequenzen teilten viele Gemeinsamkeiten, einschließlich der 12 konservierten Cystein-Reste (C1-C12) und der Aminosäuren-Blöcken, die von konservierten polymorphen Regionen flankiert werden. Die nachfolgende C2-Domäne variiert in der Länge und zeichnet sich durch vier Cysteinreste (C13-C16) und ein so genanntes "Y Motiv" aus, das eine wichtige Rolle bei der Bindungsfunktion zu haben scheint.

Die DBLβ-C2-Domäne der acht verschiedenen *P. falciparum* Feldisolate wurde je mit 16 konservierten Cystein-Resten auf der Oberfläche von COS-7-Zellen für Bindungstests mit ICAM-1 exprimiert. Die rekombinanten Konstrukte der Isolate mit klinisch schwerer Malaria präsentierten eine höhere ICAM-1-Bindungsavidität im Vergleich zu denen mit unkomplizierter Malaria, wie es aufgrund früherer Studien erwartet wurde. Eine mögliche Assoziation der ICAM-1-Bindung mit schwerer Malaria erfordert jedoch weitere Untersuchungen der Bindungskapazität an einem wesentlich größeren Patientenkollektiv mit unterschiedlichen klinischen Manifestationen und idealerweise aus verschiedenen Malariaregionen.

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APPENDIX

Appendix 1. Distribution of homology blocks (HB) in cys4 sequences from DBL1α domain, classification by Rask *et al.* using VarDom server

Sequences from severe malaria cases, gDNA (white letters).
Sequences from severe malaria cases, cDNA culture (orange letters).
Sequences from uncomplicated malaria cases, gDNA (black letters)

Sequence	Score HB3	Score HB5	Score HB2	Score S2b					Score S2c		
				HB64	HB54	HB14	HB79	HB131	HB60	HB36	HB88
Pap1.A2	15	37.8		17.8		34.3				38.1	
Pap1.A6	15.2	37.1		13.7		30.5				11.3	
Pap1.C3	15.2	35.2		10.5		10.0				44.5	
Pap1.15	15.2	34.1		19.7	11.2	31.6			12.1	43.7	
Pap1.22	15.2	40.4			11.1	37.9				50.2	
Pap1.35	15.2	34.2			15.6	25.6		10.5	10.5	40.3	
Pap2.A1	15.0	39.3			10.8	28.8	12.4			42.7	11.6
Pap2.A2	17.9	37.6		12.3		31.8	14.3	25.8		45.4	
Pap2.A3	15.2	34.9		15.0	12.8	32				50.7	
Pap2.A5		34.4				24.6				47.5	
Pap2.B1	17.9	39.3			10.8	28.8	12.4			42.7	11.6
Pap3.A11	15.0	37.6		12.3		31.8	14.3	25.8		45.4	
Pap3.B3	15.0	32.2			13.2	18.7	13.6			53.6	
Pap3.B6	15.2	36.4		14.9		29.9		18.1		48.2	
Pap3.C4	15.2	34.2	11.8			26.4			10.5	40.3	
Kal1.A1	15.0	33.8	11.5			20.0	15.1		15.3	50.2	
Kal1.A3	15.0	37.2		16.7		32.0	14.5			59.3	11.6
Kal1.B1	15.0	37.0		13.5		25.5				48.5	11.6
Kal1.B4	15.0	36.8	11.5		10.7	17.2				27.7	
Kal1.B7	18.1	37.0		14.8	18.2	33.0				45.1	
Kal1.B6	17.9	38.7	13.4	14.6	11.5	22.8			10.3	50.7	
Kal1.B8	18.1	40.2	13.4	15.3		29.1	18.2			33.2	
Kal1.B9	15.0	33.8	11.5			20.0	15.1		15.3	50.2	
Kal1.C1	12.5	40.2		16.9	19.1	34.6				51.4	
Kal1.C2	15.0	33.8				20.0	15.1		15.3	50.2	
Kal1.C5	15.0	35.8		14.7	10.3	27.0				55.5	
Kal1.C6	15.0	37.2		16.7		32.0	14.5			59.3	11.6
Kal1.C7	15.0	33.8				20.0	15.1		15.3	50.2	
Kal1.C3		36.1		13.7		24.5				38.4	
Kal1.C4	17.9	38.0	11.5	19.7	16.6	27.0				10.2	
Kal2.A1	15.2	38.2	13.4	13.4	19.4	32.3				43.5	
Kal2.A2	15.2	35.6				30.5			10.4	48.2	
Kal2.A3	15.0	36.2		16.8		27.1	13.3			50.8	11.6
Kal2.A4	15.0	35.7			13.1	20.2				27.1	
Kal2.B1	15.0	36.2		16.6	10.0	25.7				56.2	11.6
Kal2.C1	15.2	38.2		13.4	19.4	32.3				43.5	
Kal2.C2	15.2	35.6				30.5			10.4	48.2	
Kal2.C3	15.0	36.2		16.6	10.0	25.7				56.2	11.6
Kal2.C10	15.2	30.5		14.0	12.2	30.3			16.0	51.3	11.6
Kal3.A1	19.4	38.0	11.8		11.5	28.6				46.0	
Kal3.E7	17.9	44.2				15.8			10.3	15.7	
Kal4.A2	17.9	35.6				17.0				39.4	
Kal4.A3	18.1	39.1				34.6				32.0	
Kal4.A32	18.1	46.5		14.8	13.6	39.8			12.3	35.5	
Kal4.A42	15.2			14.6	13.0	34.7	16.8			30.4	
Kal4.A43	18.1	39.2		14.6	13.0	34.7	16.8			37.2	
Kal4.A52		35.2	11.8	13.7		32.3		10.1		46.4	11.6
Kal4.B2	17.9	38.7		13.8		26.2	18.2			35.4	11.6
Kal4.B5	17.9	31.9		13.5		25.5				55.0	
Kal4.B12	15.0	35.3		14.1		28.3	13.4			47.5	11.6
Kal4.B22	17.9	33.2				17.0				39.4	

Kal4.C1	18.1	38.4		11.8		34.4		12.9		37.0	11.6
Kal4.C22	15.0	32.4		14.1		28.3	13.4			47.5	11.6
Kal4.C42	17.9	37.6		12.4		29.5	16.0			32.1	
Kal5.A4	15.0	35.3		14.1		28.3	13.4			47.5	11.6
Kal5.B2	15.0	35.1				29.3			21.2	45.3	
Kal5.C3	15.2	37.0	13.4	14.8	18.2	33.0				45.1	
Kal5.C4	15.0	35.3		10.2	17.9	32.3				20.1	
Kal5.C5	12.5	37.1		15.3		28.4			11.5	43.1	
Kal5.C6	15.0	35.3		10.2	17.9	32.3				20.1	
Kal5.C7	15.0	35.3		10.2	17.9	26.3				20.1	

Appendix 2. Distribution of homology blocks (HB) in cys2 sequences from DBLα domain (classification by Rask *et al*. using VarDom server)

Sequences from severe malaria cases, gDNA (white letters).
Sequences from severe malaria cases, cDNA culture (orange letters).
Sequences from severe malaria cases, cDNA filter paper (yellow letters on red).
Sequence from an uncomplicated case, cDNA filter paper (yellow letters on black).
Sequences from uncomplicated malaria cases, gDNA (black letters)

Sequence	Score HB3	Score HB5	Score HB2	Score S2b					Score S2c		
				HB64	HB54	HB14	HB79	HB131	HB60	HB36	HB88
Pap1.A3	15.2	29.9				27.8			38.1		
Pap1.A4	15.2	36.7	11.8			30.7			48.2		
Pap1.B3	15.0	33.0				27.4			41.5		
Pap1.10	15.0	37.8				13.0			35.3		
Pap1.11	15.2	35.9			12.7	26.6			33.9		
Pap1.12	15.2	35.9			12.7	11.5			33.9		
Pap1.14	15.2	35.9			12.7	26.6			33.9		
Pap1.31	15.2	40.0		14.8	20.5	37.5			33.6		
Pap1.32	15.2	36.3	11.8		12.7	26.6			33.9		
Pap1.FP		35.9			11.6	26.6			33.9		
Pap2.C1	19.2	36.8		17.5	17.6	29.6			21.6		
Pap2.C2	18.1	36.4			11.2	26.1		18.2	47.7		
Pap2.C3	19.4	36.4	13.4		11.2	26.1		18.2	47.7		
Pap2.C4	19.4	33.1			11.1				35.4		
Pap2.FP	15.0	36.3	13.4			25.5			35.9		
Pap3.A1	15.0	38.2				21.8			44.8		
Pap3.A2	15.0	40.3				24.4			19.4		
Pap3.A8	15.0	29.5			13.3				34.2		
Pap3.A9	15.0	29.5	13.4		13.3				34.2		
Pap3.A10	15.0	29.5			13.3				34.2		
Pap3.C8	15.0	40.1		11.1		28.0			42.7		
Pap3.C9	15.0	40.1		11.1		28.0			42.7		
Pap3.FP		40.1	18.8	11.1		28.0			42.7		
Kal1.A2	15.0	34.8			11.5				39.2		
Kal1.B3	18.1	38.4		15.4	19.5	30.2			37.1		
Kal1.FP		37.4				19.1					
Kal2.B2	15.0	35.1	13.4			29.2			44.4		
Kal2.FP		37.4	14.5			27.3			43.5		
Kal3.F7	17.9	37.0				22.7			39.1		
Kal3.F10	17.9	38.6				24.6			40.8		
Kal4.C62	17.9	34.5				15.5			35.2		
Kal5.C1	15.0	34.5	13.4			15.5			43.7		
Kal5.C2	15.0	37.4				27.3			43.7		
Kal5.C8	15.0	37.4				27.3			43.7		
Kal5.FP	15.0	36.3	13.4			25.5			35.9		

Appendix 3. Distribution of homology blocks (HBs) in subdomain of DBLβ domain of field isolates using varDom server.

Sequence	Score S1	Score S2a			Score S2b						Score S2c			Score S3a			Score S3b						
	HB 53	HB 13	HB 266	HB 189	HB 146	HB 14	HB 199	HB 131	HB 174	HB 436	HB 187	HB 589	HB 183	HB 59	HB 250	HB 382	HB 119	HB 62	HB 209	HB 61	HB 30	HB 129	HB 18
Pap1	30.2	34.9				11.9					17.4			33.0				19.5		49.5			23.6
Pap2.1	30.2	39.9			12.4	24.1							26.4	40.9		11.2				57.0			25.0
Pap2.2	22.7	39.9				24.1							26.4	40.9		11.2				57.0			27.4
Pap3.1	32.0	33.7			10.9	10.9						16.1		28.5				25.4		51.1		11.6	31.6
Pap3.2	27.9	20.7		15.3	12.7	14.9		11.3		11.6				24.8	11.5		14.0	36.7		61.0			29.9
Kal1	12.0	29.9	12.7		11.9	31.3	13.4							30.7	14.7		19.4	33.8	33.9	57.1			33.5
Kal2	27.9	20.9		13.7		21.8			10.0		18.6							21.4		17.6	14.0		25.8
Kal3	27.8	29.9	12.7		11.9	31.3	13.4							30.7	14.7		19.4	33.8	33.9	57.1			33.5
Kal4	27.9	29.9	12.7		11.9	31.3	13.4							30.7	14.7		19.4		21.3	57.1			33.5
Kal5		29.9	12.7		11.9	31.3	13.4							30.7	14.7		19.4	33.8	33.9	57.1			33.5

Appendix 4. Ethical clearance for the study from Medical Faculty, Brawijaya University, Indonesia.

MINISTRY OF NATIONAL EDUCATION
BRAWIJAYA UNIVERSITY
MEDICAL FACULTY
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ETHICAL CLEARANCE

No. 008A / KEPK-FKUB / EC / I / 2009

The Ethical Committee of Medical Research Medical Faculty Brawijaya University.

With regards of protection of human rights and welfare in subject health research, and operates according to ICH-GCP guidelines and the applicable laws and regulation has carefully reviewed the proposal entitied:

" Molecular Comparison of Variant Surface Antigents in Severe falciparum Malaria (VSASM) and Variant Surface Antigents in Uncomplicated falciparum Malaria (VSAUM)"

Name of the principal investigator : dr. Erma Sulistyaningsih, M.Sc

Name of Institution : Medical Faculty Jember University

And approve the above mentioned proposal

Specified by in : Malang
On date :

Chairman

Prof. Dr. dr. Moch. Istiadjid ES, SpS, SpBS, M.Hum

Appendix 5. Pictures during field research in Indonesia.

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2. Education

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4. Publications

- Sulistyaningsih E, Fitri LE, Loescher T, Berens-Riha N, 2010. Diagnostic Difficulties with *Plasmodium knowlesi* Infection in Humans. Emerg Infect Dis (EID) 16 (6): 1033-1034 . DOI: 10.3201/eid1606.100022
- Sulistyaningsih E, Loescher T, Fleischman E, Berens-Riha N, Malaria, 2009. P. knowlesi in human in Indonesia: Kalimantan. Promed-mail
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- Sulistyaningsih E, 2007. PCR: New Age of Diagnosis and Management of Infectious Diseases. Jurnal Biomedis-FK UNEJ 1 (1).
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5. Presentations (Oral and Poster Presentations)

15-16.03.2012	DTG (Deutschen Gesellschaft fuer Tropenmedizine und International Gesundheit) – Tropical Medicine and Parasitology

	- Jahres Tagung, Heidelberg, Germany. "DBL domain expression in *Plasmodium falciparum* from Indonesia"
3-6.10.2011	7th European Congress on Tropical Medicine and International Health, Barcelona, Spain. "Expression of variant surface antigens in Indonesian Isolates from severe and uncomplicated *Plasmodium falciparum* malaria patients"
12-13.11.2010	8th Malaria Meeting, Swiss Tropical and Public Health Institute (Swiss TPH), Basel, Swiss. "Sequence diversity of Indonesian *var* genes of *Plasmodium falciparum* isolates in severe and uncomplicated malaria"
11-12.05.2010	DoktaMed 2010, the annually meeting of departments and students of Medical Faculty, LMU, Munich, Germany. "Naturally Acquired Human *Plasmodium knowlesi* Infection found in Indonesia"
03-05.05.2010	The sixth Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite, Heidelberg, Germany. "Genetic Diversity of DBLα domain of *P. falciparum var* gene in field Isolates from Indonesia"
06-07.11.2009	The Tropical Medicine 2009 Conference, the annually meeting of the German Society of Tropical Medicine and International Health (DTG), Munich, Germany "Naturally Acquired *Plasmodium knowlesi* Infection in Human beings, Indonesia"

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